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Studies on the Glomerular Filtration

I. On the Glomerular Filtration in Cases of Exudative Pleurisy, Especially on the Cause of Water Diuresis

By

Kietsu Igarashi
(五十嵐 喜悦)

(From the Medical Cinlic of Prof. F. Nakazawa, Faculty of Medicine, Tohoku University, Sendai)

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Introduction

Many factors concern the increase and decrease of the quantity of urine, but the production of it in the kidney is influenced by the following four factors; that is, the increase and the decrease of the quantity of filtration in the glomeruli, the increase and the decrease of the quantity of reabsorption in the tubuli. Thereupon I tried this experiment as I thought it clinically and pathologic-physiologically important to investigate the glomerular filtration and the tubular reabsorption concerning two or three medical diseases, the prognostic significance of which can be judged from the increase and decrease of the quantity of urine. In Part I, I report my studies on pleurisy.

It has been well known that in the case of pleurisy the quantity of urine decreases in the excretion state of exudate and increases in the absorption stage. Therefore it is very helpful for its prognosis and treatment to know the condition of water metabolism of the body and thereby to presume the acute stage or the absorption stage of the disease.

In 1924 Peyrer[1] for the first time applied water test to pleuritic patients for the purpose of finding the acute stage or the absorption stage of pleurisy, and recognized its significance. Greuel[2] also making a similar experiment and comparing it with blood sedimentation rate and temperature, emphasized its significance. This is supported by Mumme[3] and some japanese scholars f.i. Hirai.[4]-[8] But there have been very few experimental reports of water test on pleuritic patients. There is only one report made by Okazaki[9] in 1926, but the result of his examination is not very clear. Moreover in any of their water tests the relation between the change of the quantity of urine and the mechanism of urine production in the kidney is entirely unknown. Therefore I tried my water test on those pleuritic

patients who entered Prof. Nakazawa's medical clinic in and after 1946, simultaneously estimating glomerular filtration and reabsorption of the kidney and investigated its significance.

Experimental

Material and Method

Out of the patients who entered Prof. Nakazawa's medical clinic in and after 1946, I chose 30 genuine wet pleuritic patients, excluding such patients as might mistake my judgment on the result of experiment: i.e. those with pulmonary tuberculosis, those with high temperature and profuse sweating, those with edema caused by kidney or heart trouble, and those with diarrhea or constipation were eliminated. Following Volhard, I examined the diluting and concentrating power of the kidney and estimated glomerular filtration (GF) and reabsorption of the kidney (RR) per minute during the period in which secretion of urine is imagined greatest, and at the same time I calculated reabsorption percent of the kidney (RR %). As Volhard did, I gave the patient 1,000 cc. of water in 10 minutes after emptying the bladder at 8 a.m., and then I took the excretion of urine every 30 minutes before noon and every 2 hours after noon till 8 p.m., moreover adding the all excretion till 8 a.m. of the next morning. I estimated the quantity and specific gravity of this urine. As to the estimation of GF, I followed Rehberg's[10] principle; but without loading creatinine, I made a calculation by the following formula in consideration of the quantity of urine produced in an hour from 9 a.m. till 10 a.m. and the density of the urine and blood creatinine (Cu & Cp).

$$GF=\frac{U}{60}\times\frac{Cu}{Cp} \qquad RR=GF-\frac{U}{60} \qquad RR\%=\frac{RR}{GF}\times 100$$

Of course during the experiment the patient was given only dried food.

Results

As control investigation I chose 20 healthy persons who had no past history of kidney trouble. Each of the water tests is normal (Tab. I). Average GF is 167.8 cc. and RR% is 92.5 (Tab. II).

According to Nakazawa and Kusakari[11] GF of healthy persons is 160–190 cc. and RR% is 99.3 and according to Asano[12] who does not load creatinine RR% is almost the same, but GF is so small as 107.3 cc. I myself, making an estimation of 20 healthy persons without doing water test or loading creatinine, had the value of GF 129.5 cc. and RR% 98.6. Comparing this value with that of water test, I found that by water test the value of GF increases and RR% decreases. This result is significant, the probability of error being below 1%. This shows the same tendency

TABLE I

No. of case	Age	Sex	Quant. of urine in 4 hours (cc.)	Quant. of urine in first 2 hours (cc.)	Quant. of urine in 24 hours (cc.)	Maximum quant. of urine in half an hour (cc.)	Specific gravity	
							Max.	Min.
1	23	♂	1860	1385	2375	515	1,031	1,000
2	30	♂	1720	1135	2220	440	1.030	1,002
3	41	♂	1800	1460	2340	470	1,030	1,004
4	17	♂	1310	1015	1655	435	1,034	1,001
5	16	♂	1285	935	1825	350	1,030	1,004
6	25	♂	1635	1345	2050	465	1,029	1,000
7	30	♂	1770	1425	2170	490	1,030	1,000
8	27	♂	1635	1325	2045	480	1,028	1,000
9	34	♂	1455	1095	1840	410	1,030	1,002
10	38	♂	1270	950	1760	360	1,028	1,002
11	37	♀	1075	855	1635	420	1,027	1,001
12	22	♀	1250	945	1600	350	1,030	1,002
13	31	♀	1390	745	1735	360	1,030	1,004
14	20	♀	1345	1135	1675	460	1,032	1,001
15	20	♀	1405	1140	1825	580	1,028	1,000
16	23	♀	1245	980	1605	420	1,030	1,001
17	22	♀	1500	1100	1815	400	1,030	1,001
18	45	♀	1170	860	1660	390	1,028	1,002
19	38	♀	1295	755	1750	360	1,030	1,001
20	25	♀	1220	910	1640	400	1,028	1,001
Average			1432	1075	1861	427		

with the result once obtained by Hiratsuka[18] under Prof. Nakazawa's direction.

I divided the result of the water test on the pleuritic patients into 3 groups. The first group consists of those that are in the acute stage according to the clinical picture, the quantity of urine in 4 hours being much less than the dosis of drinking water. The second group consists of those that are still far from absorption stage though there is no symptom of being in clinically acute stage, the quantity of urine in 4 hours being nearly the same as the dosis of drinking water. The third group consists of those that are already in clinically absorption stage, the quantity of urine in 4 hours being much more than the dosis of drinking water.

1. *The first group*: *acute stage* (Tab. III, IV)

The excretion of water is prevented; the average quantity of urine in 4 hours is 568 cc., which is less than $\frac{1}{3}$ of the normal quantity. The excretion of water reaches maximum within the first 2 hours, but the maxi-

TABLE II

No. of case	Age	Sex	Urine an hour		Creatinine mg/dl		Quant. of urine per min. cc.	GF cc.	RR cc.	RR %
			Quant. of urine (cc.)	Specific gravity	Urine	Plasma				
1	23	♂	980	1,000	10,51	0,80	16,3	214,0	197,7	92,3
2	30	♂	810	1,002	9,38	0,53	13,5	238,9	225,4	94,3
3	41	♂	905	1,004	10,40	0,85	15,0	181,1	166,1	91,6
4	17	♂	650	1,001	8,13	0,67	10,8	131,0	120,2	91,7
5	16	♂	590	1,004	12,45	0,61	9,8	187,7	177,9	94,7
6	25	♂	890	1,000	7,59	0,77	14,8	145,8	131,0	89,8
7	30	♂	920	1,000	8,26	0,82	15,4	155,1	139,7	90,0
8	27	♂	850	1,001	9,06	0,78	14,1	163,7	149,6	91,3
9	34	♂	720	1,002	9,53	0,62	12,0	184,2	172,1	93,4
10	38	♂	600	1,002	11,75	0,65	10,0	180,7	170,7	94,4
11	37	♀	620	1,001	10,50	0,83	10,3	130,3	120,0	92,0
12	22	♀	595	1,004	14,10	0,78	9,9	178,9	169,0	94,4
13	31	♀	590	1,004	9,34	0,60	9,8	152,5	142,7	93,5
14	20	♀	810	1,001	7,98	0,63	13,5	171,0	157,5	92,1
15	20	♀	960	1,000	7,19	0,65	16,0	176,9	160,9	90,9
16	23	♀	680	1,002	10,32	0,76	11,3	153,4	142,1	92,6
17	22	♀	750	1,001	9,75	0,72	12,5	167,9	155,4	92,5
18	45	♀	650	1,002	11,06	0,81	10,8	147,4	136,6	92,6
19	38	♀	580	1,002	11,07	0,75	11,3	141,6	132,0	93,2
20	25	♀	630	1,001	10,31	0,68	10,5	154,6	144,1	93,2
Average			739		9,93	0,71	12.4	167,8	155,4	92,5

TABLE III

No. of case	Age	Sex	Quant. of urine in 4 hours (cc.)	Quant. of urine in first 2 hours (cc.)	Quant. of urine in 24 hours (cc.)	Maximum quant. of urine in half an hour (cc.)	Specific gravity	
							Max.	Min.
1	19	♂	205	124	815	35	1,032	1,024
2	25	♂	495	285	1250	135	1,032	1,014
3	29	♀	580	395	1035	150	1,030	1,012
4	19	♀	585	485	745	225	1,032	1,002
5	59	♀	590	385	990	185	1,024	1,008
6	32	♀	740	630	1080	240	1,030	1,006
7	21	♀	780	470	1205	215	1,030	1,006
Average			568	396	1017	169		

TABLE IV

No. of case	Age	Sex	Urine an hour		Greatinine mg/dl		Quant. of urine per min. cc.	GF cc.	RR cc.	RR %
			Quant. of urine cc.	Specific gravity	Urine	Plasma				
1	19	♂	62	1,024	111,67	1,14	1,0	97,9	96,9	98,8
2	25	♂	135	1,014	57,33	1,27	2,2	99,3	97,1	97,6
3	29	♀	270	1,012	18,06	0,85	4,5	95,6	91,1	95,3
4	19	♀	330	1,002	17,59	0,78	5,5	124,0	118,5	95,5
5	59	♀	125	1,010	12,66	0,59	2,0	42,9	40,9	95,3
6	32	♀	460	1,006	14,83	0,88	7,6	128,0	120,4	94,0
7	21	♀	325	1,006	22,53	0,96	5,4	126,7	121,3	95,7
Average			244		36,38	0,92	4,0	102,0	98,0	96,0

mum quantity of urine in half an hour averages 169 cc., which shows less than 1/3 of the dosis of drinking water. In 3 cases the quantity of urine in 24 hours does not reach the dosis of drinking water, and in 4 cases it shows delay, incomplete reaction. The minimum specific gravity except 1 case is above 1,004 and the diluting power of the kidney is prevented, and the maximum specific gravity is normal except 1 case. Besides, there is 1 case that excretes 20–30 cc. of urine with high specific gravity every 30 minutes all through the course. Next, average GF is 102.0 cc., and RR% is 96.0 which shows that though GF decreases, RR% increases. This fact proves that, in the water test, the diluting power is prevented and that the concentrating power is normal. The decrease of GF and the increase of RR% show statistically that the probability of error is below 1% and so it is significant. Therefore it is clear that though the oliguria is mainly due to the decrease of GF, it is partly due to the increase of RR.

2. *The second group: middle stage* (Tab. V, VI)

The excretion of water is considerably better than that in the acute stage. The quantity of urine in 4 hours averages 1080 cc., which is almost normal. In 4 cases the maximum quantity of urine in half an hour is above 1/3 of the dosis of drinking water. In 5 cases the quantity of urine in 24 hours is above 1,500 cc., and shows delay, excessive reaction; and in 4 cases it shows delay, incomplete reaction. The minimum specific gravity is a little prevented in 3 cases, but the maximum specific gravity is normal respectively. GF averages 144.2 cc., which is considerably more than that in the acute stage, but is less than the normal value. This decrease of GF shows statistically that the probability of error is below 5% and so it is significant. RR averages 93.0% and so it is nearly normal. Therefore in the middle stage, though GF decreases, RR suppression is almost

TABLE V

No. of case	Age	Sex	Quant. of urine in 4 hours (cc.)	Quant. of urine in first 2 hours (cc.)	Quant. of urine in 24 hours (cc.)	Maximum quant. of urine in half an hour (cc.)	Specific gravity	
							Max.	Min.
1	19	♂	890	775	1435	330	1,032	1,003
2	24	♀	913	747	1186	305	1,032	1,006
3	22	♀	1045	915	1220	350	1,034	1,003
4	46	♂	1055	935	1580	350	1,031	1,002
5	52	♂	1066	883	1691	345	1,027	1,003
6	22	♂	1090	640	1357	270	1,033	1,005
7	29	♀	1137	837	1501	405	1,036	1,002
8	46	♂	1235	915	1775	330	1,028	1,002
9	25	♂	1295	970	2100	315	1,034	1,008
Average			1080	846	1538	333		

TABLE VI

No. of case	Age	Sex	Urine an hour		Creatinine mg/dl		Quant. of urine per min. (cc.)	GF cc.	RR cc.	RR %
			Quant. of urine (cc.)	Specific gravity	Urine	Plasma				
1	19	♂	515	1,003	15,44	0,87	8,5	150,8	142,3	94,3
2	24	♀	535	1,004	14,23	1,24	8,9	102,1	93,2	91,2
3	22	♀	680	1,004	11,47	0,72	11,3	180,0	168,7	93,7
4	46	♂	670	1,002	10,11	0,75	11,1	149,6	138,5	92,5
5	52	♂	545	1,003	14,15	0,81	9,0	157,2	148,2	94,4
6	22	♂	530	1,005	12,23	0,90	8,8	119,5	110,7	92,6
7	29	♀	560	1,003	12,07	0,65	9,3	172,7	163,4	94,6
8	46	♂	625	1,002	9,15	0,64	10,4	148,6	138,2	93,0
9	25	♂	625	1,008	9,16	0,81	10,4	117,6	107,2	91,1
Average			587		12,00	0,82	9,7	144,2	134,5	93,0

normal and so it seems that comparatively much quantity of urine is excreted, though not satisfactorily.

3. *The third group*: *absorption stage* (Tab. VII, VIII)

The excretion of water is very good. The quantity of urine in 4 hours averages 1423 cc. and the most part of it is excreted within the first 2 hours. Besides, in some cases good excretion continues even two hours longer. The maximum quantity of urine in half an hour is over 1/3 of the dosis of drinking water in every case. The quantity of urine in 24 hours is over 1,640 cc., showing excessive excretion reaction. The mini-

TABLE VII

No. of case	Age	Sex	Quant. of urine in 4 hours (cc.)	Quant. of urine in first 2 hours (cc.)	Quant. of urine in 24 hours (cc.)	Maximum quant. of urine in half an hour (cc.)	Specific gravity	
							Max.	Min.
1	25	♀	1300	895	1640	370	1,031	1,006
2	24	♂	1340	1100	2085	480	1,031	1,001
3	52	♂	1360	930	1890	385	1,030	1,003
4	21	♀	1360	1055	1790	450	1,031	1,001
5	21	♂	1370	1195	1695	420	1,033	1,002
6	19	♂	1375	1125	1920	610	1,032	1,003
7	25	♀	1390	1045	1790	580	1,032	1,010
8	32	♀	1400	1090	1735	350	1,032	1,002
9	29	♀	1405	1010	2015	390	1,026	1,006
10	27	♂	1410	960	1890	450	1,028	1,004
11	29	♀	1475	960	1965	390	1,028	1,002
12	48	♂	1500	1170	1940	335	1,028	1,003
13	22	♂	1530	1330	2000	600	1,038	1,004
14	25	♂	1730	965	2495	405	1,031	1,005
Average			1423	1059	1921	443		

TABLE VIII

No. of case	Age	Sex	Urine an hour		Creatinine mg/dl		Quant. of urine per min. (cc.)	GF cc.	RR cc.	RR %
			Quant. of urine (cc.)	Specific gravity	Urine	Plasma				
1	25	♀	700	1,006	8,23	0,65	11,6	146,8	135,2	92,0
2	24	♂	650	1,002	15,23	0,75	10,8	219,3	208,5	95,0
3	52	♂	635	1,003	11,74	0,72	10,5	171,2	160,7	93,8
4	21	♀	770	1,001	9,91	0,66	12,8	192,1	179,3	93,3
5	21	♂	700	1,002	11,56	0,78	11,6	171,9	160,3	93,2
6	19	♂	810	1,003	10,72	0,71	13,5	203,8	190,3	93,3
7	25	♀	905	1,010	11,50	0,68	15,0	253,6	238,6	94,0
8	32	♀	690	1,002	8,06	0,60	11,5	154,4	142,9	92,5
9	29	♀	625	1,006	9,69	0,69	10,4	146,0	135,6	92,8
10	27	♂	740	1,004	14,64	0,82	12,3	219,6	207,3	94,3
11	29	♀	750	1,002	10,83	0,64	12,5	211,5	199,0	94,0
12	48	♂	635	1,003	12,19	0,70	10,5	182,8	172,3	94,6
13	22	♂	985	1,004	8,52	0,60	16,4	232,8	216,4	92,9
14	25	♂	610	1,005	10,07	0,62	10,1	164,0	153,9	93,8
Average			729		10,92	0,68	12,1	190,7	178,6	93,5

mum specific gravity is slightly prevented in 3 cases, but the maximum is normal in every case. GF averages 190.7 cc., which is more than normal. This fact shows statistically that the probability of error is below 5% and so it is significant. RR% averages 93.5 and so it is a little higher than normal. Therefore polyuria in the absorption stage is mainly due to the increase of GF and the suppression of RR.

I can summarize the above results as follows. In the acute stage the excretion of water is poor, and diluting power is prevented. This oliguria is due to the decrease of GF and the slight suppression of RR. In the middle stage the excretion of water is a little better, and almost normal. In this stage GF decreases, but as RR is suppressed, the excretion of sufficient quantity of water is possible. In the absorption stage the excretion of water is excess owing to the increase of GF and the suppression of RR, though in a few cases the diluting power is a little prevented.

To confirm the above results, I made estimations in several periods of 3 patients who were in hospital for a long time from the beginning of their disease. The first case: 19 years old male patient who entered hospital at the beginning of disease, with a clinical picture indicating the acute stage. As Tab. IX, X show, the quantity of urine increases as the symptom grows slighter. The diluting and concentrating power, prevented at first, becomes normal again. GF, very little at first, grows remarkable afterward. RR suppression, also very little at first, becomes great afterward. The second and third cases show the same process though the grade of each patient is different. That is, in the acute stage of pleurisy the result of water test is bad, but grows better as it progresses, and in the absorption stage it becomes excessive excretion. Therefore it is clear that in the acute stage the change of the quantity of urine is due to the decrease of GF and the slight suppression of RR; and in the absorption stage it is due to the increase of GF and the suppression of RR.

Consideration

Concerning diuresis by water test, Underhill and Pack[14)-15)] etc. made their experiments on dogs and stated that they could not recognize any hydremia, but I think it is unreasonable in common sense. Hashimoto[16)-20)] and many others proved hydremia by animal and human experiment, concluding that in time of water diuresis, extra-renal total hydremia plays an important part. Concerning the influence of this hydremia upon GF and RR, there are many reports. Poulsson,[19)] Rehberg, Holten[21)-25)] etc. say that though GF does not increase, RR decreases remarkably. Chrometzka and Unger[26)-27)] etc. say that GF increases temporally, but decreases after all and that RR suppression becomes remarkable. However, Hiratsuka[18)] recognizes in his experiments on dogs that not only the decrease of RR

TABLE IX

No. of case	Age	Sex	Quant. of urine in 4 hours (cc.)	Quant. of urine in first 2 hours (cc.)	Quant. of urine in 24 hours (cc.)	Maximum quant. of urine in half an hour (cc.)	Specific gravity	
							Max.	Min.
1	19	♂	205	124	815	35	1,032	1,024
			890	775	1435	330	1,027	1,003
			1375	1125	1920	610	1,032	1,003
2	25	♂	495	285	1250	135	1,032	1,014
			1295	970	2100	315	1,034	1,008
			1730	965	2495	405	1,031	1,005
3	29	♀	580	395	1035	150	1,030	1,012
			1137	837	1501	405	1,036	1,002
			1475	960	1965	390	1,028	1,002

TABLE X

No. of case	Age	Sex	Urine an hour		Creatinine mg/dl		Quant. of urine per min. (cc.)	GF cc.	RR cc.	RR %
			Quant. of urine (cc.)	Specific gravity	Urine	Plasma				
1	19	♂	62	1,024	111,67	1,14	1,0	97,9	96,9	98,8
			515	1,003	15,44	0,87	8,5	150,8	142,3	94,3
			810	1,003	10,72	0,71	13,5	203,8	190,3	93,3
2	25	♂	135	1,014	57,33	1,27	2,2	99,3	97,1	97,6
			625	1,008	9,16	0,81	10,4	117,6	107,2	91,1
			610	1,005	10,07	0,62	10,1	164,0	153,9	93,8
3	29	♀	270	1,012	18,06	0,85	4,5	95,6	91,1	95,3
			560	1,003	12,07	0,65	9,3	172,7	163,4	94,6
			750	1,002	10,83	0,64	12,5	211,5	199,0	94,0

but also the increase of GF is the cause of water diuresis. Like this, the theories do not agree, as to the cause of diuresis in the water test.

I estimated GF and RR of healthy persons neither making water test nor loading creatinine and obtained the value of GF 129.5 cc. and RR% 98.6, but estimating GF and RR when the quantity of urine is maximum by loading water, I had the value of GF 167.8 cc. and RR% 92.5. This result is statistically significant as I have already stated. Therefore I cannot but support Hiratsuka's theory that the increase of the quantity of urine by water test is caused not only by the RR suppression but also by the increase of GF.

As is well known, there is oliguria in the acute stage and polyuria in the absorption stage of pleurisy, so by estimating the daily quantity of urine, we can imagine the condition of pleura to some degree. But when the absorption of exudate lasts long, there is no remarkable change in the

daily quantity of urine, and so it is not always possible to imagine the condition of pleura. However, Peyrer and others tested pleuritic patients by Volhard's water test and estimating the quantity of urine in 4 hours and 12 hours, they found the quantity is very little compared with the dosis of drinking water in the acute stage, and remarkably much in the absorption stage; which, they asserted, is helpful for deciding when pleurocentesis should be done, and consequently gives some therapeutic effect. But they mainly treated of the change of the quantity of urine and did not refer to the mechanism of urine in the kidney by water test. They did not make clear whether it is owing to the decrease of GF or to the extreme acceleration of RR though GF is normal, that the quantity of urine in 4 hours is so much less than the dosis of drinking water in the acute stage. Nor did they make clear whether it is owing to increase of GF or to the suppression of RR or to both, that the quantity of urine in 4 hours is so excessive in the absorption stage. These problems have been solved by my experiment.

Next, I tried to make a theoretical examination on the way of cure from the result of my experiment. There is no doubt that the change of GF and RR in pleurisy is due to the abnormality of exudation and resorption at the very locality where pleurisy exists, but judging from the fact that in many pleuritic patients we sometimes find temporary nephritic symptom caused by tubercular allergy, I think it can be concluded that in the case of pleurisy there occurs an abnormality of the function of kidney as one of the symptoms of allergy, which causes the change of GF and RR.

Now, as oliguria in the acute stage occurs on account of the decrease of GF and the slight suppression of RR, we ought to try to increase GF and to decrease RR% for the purpose of increasing the quantity of urine. Consequently we are suggested to dose Euphyllin which is believed to expedite diuresis, or Salyrgan which is believed to suppress RR. But as Nonnenbruch[28] says, the genesis and absorption of pleural exudate is not due to the total water metabolism but to the partial condition of the patient, and when the inflammation is severe, it is impossible to change oliguria into polyuria and moreover it is more reasonable to keep the kidney quiet than to stimulate it which is very sensitive on account of allergy; so I do not think it is very effective to dose diuretic carelessly in such a case. Besides, with the exception of Trousseau's indication, there are pro and con views as to the removal, by puncture, of the liquid produced in the acute stage. Mumme and others say, according to their experiments, that pleurocentesis is not advisable in the acute stage when oliguria is found by water test, and I myself have the same opinion, because in the acute stage the liquid is easily produced again if it removed by puncture. Again I

found that the water test in the acute stage does not show any side action, though I had an experience of finding the decrease of the quantity of urine for one or two days after the test, which however became normal soon again.

In the middle stage there is a decrease of GF though the suppression of RR is enough, so that administration of Euphyllin can be expected as it is helpful to increase GF. Besides, I have an interesting experience that soon after the trying of water test in this stage the quantity of urine increases remarkably sometimes, because the water test itself becomes the means of water thrust test, expediting reabsorption so much.

Finally, in the absorption stage there is sometimes a want of RR suppression, though GF is enough, so by suppressing RR more, greater quantity of urine can be produced. Therefore the administration of Salyrgan is reasonable, as it is helpful to suppress RR. In this case too, there are some cases in which water test itself becomes water thrust test, which therefore needs no other means.

Conclusion

In the acute stage of wet pleuritic patients the result of water test is bad. The oliguria in this stage is due to the decrease of glomerular filtration and to the decrease of the suppression of the kidney reabsorption. In the absorption stage the excretion of urine is excess. This is due to the excessive increase of glomerular filtration and to the suppression of reabsorption of the kidney. From this foundation I have referred to the way of cure.

Summarising this, I can say that in the clinical treatment of pleuritic patients the water test and the estimation of glomerular filtration and reabsorption of the kidney do not give any unpleasant influence to the patients, but help to decide whether they are in the acute stage or in the absorption stage, and consequently give suggestions to the way of cure, and moreover give sometimes a good influence to them as means of water thrust test.

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Studies on the Glomerular Filtration

II. On the Glomerular Filtration in Cases of Polyuria and Oliguria

By

Kietsu Igarashi
(五十嵐 喜悦)

(From the Medical Clinic of Prof. F. Nakazawa, Faculty of Medicine, Tohoku University, Sendai)

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Introduction

The increase and decrease of the quantity of urine gives one of the most important suggestions in the way of medical treatment of any disease, but as there are many complicated factors concerning it, it is impossible to explain it simply. This is mainly due to intrarenal factors and above all, due to the change of renal blood flow.[1)-9)] Nakazawa, Izumi and Seki[10)] investigated the increase and decrease of the quantity of urine concerning cardiovascular diseases and there are also some reports made by Lassen[11)-14)] etc. almost at the same time. Again, as it is well known there is remarkable diuresis in the convalescent stage of infective disease. As to this phenomenon Inoue[15)] of Prof. Nakazawa's medical clinic made a report about the mechanism of diuresis in the convalescent stage of typhoid fever.

However, observing several degrees of polyuria and oliguria in several diseases, the relation between this and the glomerular filtration and tubular reabsorption of the kidney is not very clear. Therefore in many cases of polyuria and oliguria I investigated the relation between the quantity of urine and the glomerular filtration and tubular reabsorption of the kidney without loading creatinine, and tried to contribute to the therapy of oliguria and to the study of mechanism of urine production in the kidney.

Experimental

Material and Method

The material of experiment were chiefly the patients who entered Prof. Nakazawa's medical clinic with polyuria or oliguria. The method was as follows. On the day of experiment the patient is not given any

water or food. After urination at 8 a.m. the patient is loaded with 100 cc. of water and I estimated the quantity of urine, and creatinine in the blood and urine, and according to the formula stated in part I, I calculated glomerular filtration (GF) and tubular reabsorption (RR) and reabsorption percent (RR%).

Results of Experiments

1. *In the case of polyuria*

In the group of the diseases which have no essential relation with the quantity of urine, 20 cases excrete more than 2000 cc. of urine; 6 cases that once had oliguria owing to organic heart disease show remarkable increase of the quantity of urine after therapy; 16 cases of peritonitis or liver cirrhosis and other diseases show remarkable polyuria in the course of therapy; i.e. I made 42 estimations in various cases of polyuria.

TABLE I

No. of case	Age	Sex	Diagnosis	Urine an hour		Creatinine mg/dl		Quant. of urine per min. cc.	GF cc.	RR cc.	RR %
				Quant. of urine cc.	Specific gravity	Urine	Plasma				
1	23	♂	Neurasthenia	130	1,014	45,41	0,72	2,1	132,4	130,3	98,4
2	25	♂	"	125	1,015	57,88	0,76	2,0	152,3	150,3	98,6
3	26	♂	"	104	1,019	72,86	0,72	1,7	170,0	168,3	99,0
4	38	♂	"	120	1,010	55,63	0,73	2,0	152,4	150,4	99,4
5	20	♀	"	165	1,011	28,00	0,55	2,7	137,0	134,3	98,0
6	45	♀	"	97	1,018	49,27	0,65	1,6	121,2	119,6	98,6
7	20	♀	Hysteria	125	1,010	46,03	0,70	2,0	131,5	129,5	98,4
8	22	♀	"	110	1,014	38,87	0,71	1,8	98,5	96,7	98,1
9	37	♀	"	120	1,012	38,92	0,65	2,0	119,7	117,7	98,3
10	38	♂	Bell's Palsy	100	1,016	50,37	0,68	1,6	118,8	117,2	98,6
11	41	♀	"	150	1,015	37,25	0,70	2,5	131,5	129,0	98,1
12	42	♀	"	98	1,016	44,27	0,56	1,6	126,4	124,8	98,7
13	22	♀	Cardiac neurosis	160	1,016	24,89	0,63	2,6	102,7	100,1	97,4
14	30	♀	"	150	1,007	39,94	0,61	2,5	163,5	161,0	98,4
15	40	♂	"	103	1,016	71,30	0,79	1,7	153,4	151,7	98,5
16	25	♂	"	380	1,005	19,60	0,77	6,3	160,3	154,0	96,0
17	31	♂	"	160	1,014	46,23	0,75	2,6	160,2	157,6	98,3
18	44	♂	"	154	1,016	84,03	0,86	2,5	244,2	241,7	98,9
19	54	♂	"	135	1,012	46,74	0,68	2,2	151,3	149,1	98,4
20	58	♂	"	270	1,010	21,96	0,67	4,5	147,4	142,9	96,8
Average				148		45,97	0,67	2,4	143,7	141,3	98,2
Average of healthy person				102		54,70	0,71	1,6	129,5	127,9	98,6

Table I shows the estimation value of 20 cases of neurasthenia etc.. 2 or 3 cases show normal or less normal value of GF, but as most of them show more than normal value, the average is 143.7 cc. RR is different in each case, but as the average is 141.3 cc., its value is comparatively high. These are not sufficient enough to show the urine production mechanism in the kidney, but only 3 cases show RR value below 98%, (No. 13, 16, 20), while all the others show more than 98%. If less than 98% is considered to be caused by the suppression of reabsorption, the majority in Table I are to be said normal in reabsorption. Therefore the polyuria in these

TABLE II

No. of case	Age	Sex	Diagnosis	Urine an hour		Creatinine mg/dl		Quant. of urine per min. cc.	GF cc.	RR cc.	RR %
				Quant. of urine cc.	Specific gravity	Urine	Plasma				
1	53	♂	Aortic stenosis	165	1,012	51,05	0,85	2,5	150,1	147,8	98,4
2	19	♂	Mitral heart failure	230	1,012	35,21	0,95	3,8	140,8	137,0	97,3
3	19	♂	„	215	1,012	26,68	0,66	3,5	141,4	137,9	97,5
4	39	♀	„	145	1,010	26,72	0,67	2,4	95,7	93,3	97,3
5	21	♂	„	215	1,007	28,88	0,62	3,5	163,0	159,5	97,8
6	57	♀	Mitral insfficiency	155	1,006	23,70	0,65	2,5	91,0	88,5	97,2
7	19	♂	Nephrosis	160	1,011	25,39	0,67	2,5	94,7	92,2	97,3
8	20	♀	„	205	1,012	25,54	0,57	3,4	152,3	148,9	97,7
9	20	♀	Peritonitis	165	1,011	38,00	0,65	2,7	157,7	155,0	98,2
10	28	♂	„	147	1,010	41,06	0,79	2,4	124,7	122,3	98,0
11	49	♀	Cirrhosis of liver	160	1,010	24,96	0,80	2,5	76,6	74,1	96,7
12	32	♂	Kala-Azar	185	1,010	29,50	0,82	3,0	107,9	104,9	97,2
13	45	♂	Hodgkin's disease	130	1,010	34,30	1,11	2,1	64,8	62,7	96,7
14	46	♂	Bacillary dysentery	200	1,012	36,80	0,93	3,3	130,5	127,2	97,4
15	29	♀	„	270	1,010	18,90	0,87	4,5	97,9	93,4	95,3
16	34	♂	Weil's disease	140	1,018	66,75	0,73	2,3	210,3	208,0	98,9
17	51	♀	Essential hypertension	110	1,010	32,56	0,70	1,8	83,3	81,9	97,8
18	44	♀	„	112	1,010	30,78	0,68	1,8	81,4	79,6	97,7
19	45	♂	Secondary contracted kidney	105	1,007	50,73	2,05	1,7	42,0	40,3	95,9
20	66	♂	„	116	1,012	43,44	0,83	1,9	99,4	97,5	98,0
21	45	♂	Genuine contracted kidney	180	1,010	29,37	1,12	3,0	78,6	75,6	96,2
22	52	♀	„	100	1,020	34,78	0,78	1,6	71,3	69,7	97,7
Average				164		34,32	0,84	2,6	116,6	114,0	97,3
Average of healthy person				102		54,70	0,71	1,6	129,5	127,9	98,6

cases must be due to the increase of glomerular filtration. This is the result of hydremia caused by the great quantity of water taken before the experiment owing to neurotic thirst, or due to the increase of renal blood flow caused by neurotic acceleration of heart function, but I believe it is not due to the abnormality of tubular function.

Table II shows the estimation values of 22 cases of organic heart disease or of other disease that have essential relation with polyuria. GF is below 100 in the majority out of which 1 case of secondary contracted kidney (No. 19), 1 case of genuine contracted kidney (No. 21), 1 case of diabetis mellitus (No. 22) show especially remarkable decrease of GF. RR generally increases and decreases in accordance with GF, and RR% is below 98 in 17 cases, majority of which, therefore show remarkable suppression of reabsorption. However though there is remarkable polyuria in each case, remarkable increase of GF is found only in one case (No. 16) of Weil's disease. Therefore concerning the above diseases, it is clear that the polyuria caused by therapy or convalescent stage owes its mechanism not so much to the increase of GF as to the suppression of tubular reabsorption. This proves that there is an essential difference between the polyuria in Table I and that in Table II. In cases of Table II, though good circulation can be considered of course, the function of tubular epitherium of the kidney is supposed to recover and actively suppress tubular reabsorption.

Concerning the urine production mechanism, there have been many theories, but nowadays the theory on glomerular filtration and tubular reabsorption advocated by Cushny etc. is most highly estimated. And there are many theories as to the function of tubular epitherium of the kidney. Some say tubular epitherium reabsorbs actively, while Yamaguchi[16] asserts that those epitherium actively suppresses reabsorption and helps the excretion of water and urine. I should say that tubular epitherium of the kidney reabsorbs such necessary things as sugar and actively prevents from reabsorption such waste materials as urea, uric acid and creatinine; i.e. I can say that tubular epitherium actively suppresses reabsorption in the way of its function. Of course this is a hypothesis, but I want to examine whether this hypothesis is helpful or not in the following cases of oliguria.

2. *In the case of oliguria*

As Table III shows, in 12 cases of organic heart disease GF is 50 or below it except in 4 cases (No. 3, 4, 8 and 9). RR is near the value of GF, but RR% is below 98 in only 1 case, and reabsorption percent is normal or over it. Therefore oliguria in this cases is due to the remarkable decrease of GF, but the acceleration of RR is supposed to be partly the case

of it. I think this is mainly due to the decrease of renal blood flow, but at the same time the suppression of function of reabsorption in the tubuli is prevented to some degree.

TABLE III

No. of case	Age	Sex	Diagnosis	Urine an hour		Creatinine mg/dl		Quant. of urine per min. cc.	GF cc.	RR cc.	RR %
				Quant. of urine cc.	Specific gravity	Urine	Plasma				
1	59	♂	Mitral insufficiency	40	1,012	73,54	2,09	0,6	21,1	20,5	97,1
2	20	♂	Pericarditis	35	1,026	94,58	0,91	0,5	51,9	51,4	99,0
3	25	♂	Septic acute endocarditis	30	1,024	135,33	1,09	0,5	62,0	61,5	99,1
4	22	♀	Mitral insufficiency	35	1,022	110,21	0,91	0,5	60,5	60,0	99,1
5	22	♀	"	26	1,024	106,91	0,77	0,4	55,5	54,1	99,2
6	57	♀	Mitral stenosis	25	1,020	100,24	0,83	0,4	48,3	47,9	99,1
7	40	♀	"	20	1,026	175,97	0,88	0,3	59,9	59,6	99,6
8	24	♀	Aortic insufficiency	35	1,017	79,48	0,50	0,5	79,4	78,9	99,3
9	32	♀	Mitral stenosis	38	1,023	93,57	0,58	0,6	96,7	96,1	99,3
10	42	♀	Septic acute endocarditis	35	1,025	118,83	1,63	0,5	36,4	35,9	98,6
11	19	♂	Mitral insufficiency	30	1,018	64,13	0,78	0,5	41,1	40,6	98,7
12	53	♂	Aortic stenosis	32	1,020	147,35	0,94	0,5	78,5	78,0	99,3
13	67	♂	Genuine contracted kidney	30	1,026	128,97	0,98	0,5	63,7	63,2	99,2
14	45	♀	Secondary contracted kidney	35	1,020	83,05	0,75	0,5	55,3	54,8	99,0
15	67	♀	"	35	1,025	80,98	0,81	0,5	49,9	49,4	98,9
16	21	♂	Nephrosis	20	1,032	262,62	1,09	0,3	72,1	71,8	99,5
17	19	♀	"	40	1,024	120,34	0,72	0,6	100,2	99,6	99,5
18	21	♂	"	35	1,022	107,69	1,03	0,5	52,2	51,7	99,0
19	31	♀	Acute pancreatitis	30	1,030	145,13	0,84	0,5	86,3	85,8	99,4
20	47	♂	"	40	1,020	86,55	0,76	0,6	68,3	67,7	99,1
21	26	♂	Atony of stomach	40	1,021	108,64	0,79	0,6	82,5	81,9	99,2
22	34	♂	"	38	1,025	142,11	0,86	0,6	99,1	98,5	99,3
23	55	♀	"	41	1,026	78,83	0,75	0,6	63,0	62,4	99,0
24	42	♂	"	40	1,018	98,52	0,72	0,6	82,1	81,5	99,2
Average of healthy person				102		54,70	0,71	0,6	129,5	127,9	98,6

Next, examining genuine and secondary contracted kidney, and nephrosis in the same way, I found the remarkable decrease of GF and the acceleration of tubular reabsorption. This phenomenon is supposed to be caused by the decrease of renal blood flow on account of the organic

change of the kidney, and the function of tubular epitherium declines, as it is clear from the histopathologic point of view, which therefore makes tubular reabsorption insufficient.

Acute pancreatitis too shows nearly the same symptom, and this is supposed to be the result of the prevention of the reabsorptive suppression caused by the abnormality of renal blood flow which decreases GF, together with a disorder of tubular epitherium caused by toxin.

In the case of intense atony of stomach GF and RR decreases to some degree and RR% is almost the same as it is in other cases of oliguria, but is a little higher. In this case the prevention of water absorption in intestine is supposed to cause the decrease of GF, but it is impossible to imagine that the organic disorder of cell lowers the reabsorbtive function of tubular epitherium. Therefore I cannot but conclude that this is due to the abnormality of the function of the kidney cell caused by the disorder of autonomic nervous system which rules the function of the kidney cell. The reason is that the mutual relation between stomach atony and autonomic nervous system exists. As I have stated, polyuria and oliguria are mainly due to the increase and decrease of GF, but there is no doubt that the quantity of reabsorption in tubuli has relativity with it, which influences the quantity of urine. In this case, as I have stated, it is more convenient for explanation to think that the suppression of reabsorption in tubuli means the active function of tubular epitherium; i.e. in some pathological condition oliguria occurs owing to the prevention of reabsorbtive function of tubuli, and I should believe that in some cases if tubular epitherium recovers its function as the disease grows slighter and actively suppresses reabsorption, it will cause diuresis consequently.

Conclusion

In cases of polyuria or oliguria caused by various diseases I estimated glomerular filtration and tubular reabsorption without loading creatinine.

1) The polyuria of the diseases that have no essential relation with the increase of the quantity of urine is mainly due to the increase of glomerular filtration.

2) The polyuria of the diseases that have essential relation with polyuria is mainly due to the suppression of tubular reabsorption.

3) Oliguria of various diseases is mainly due to the remarkable decrease of glomerular filtration, but partly due to the slight suppression of tubular reabsorption.

Judging from the result of this experiment, the therapy of oliguria must be done in consideration of two factors; i.e. one is to remove the prevention of circulation or to increase renal blood flow by physical treatment for the purpose of increasing the glomerular filtration; and the other

is to recover the function of tubular epitherium of the kidney by protecting it in order to suppress the tubular reabsorption.

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F. Nakazawa.

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Identification of Methyl Glyoxal from Human Urine, Preliminary Report

by

Hiroshi Wako
(若　生　　宏)

(From the Department of Pediatrics, Faculty of Medicine, the Tohoku University, Sendai. Director: Prof. A. Sato)

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Since the publication of Meyerhof scheme, the position of methyl glyoxal in carbohydrate metabolism has lost in favor in general. But Prof. Sato[1] has always been of the opinion that the cause of infantile beriberi[2] is methyl gloyoxal or like substance in human milk since the publication of Arakawa's reaction[3] in our Laboratory. Recently, I have succeeded in isolating methyl glyoxal[4] from human milk in strictly chemical way.

Now I was able to identify it from human urine. About 1,000 cc. of mother's urine was treated by ether with Soxhlet extraction apparatus for 5～6 hours. Ether was driven away from the extract and 20 cc. of water were added. Then steam distillation followed. The fraction was received in a receiver containing 0.1 % 2,4-dinitrophenylhydrazine solution. Each drop caused yellowish precipitates immediately. Two more receivers were used till the distiltation was completed. In a few days reddish precipitates occurred which showed blue violet reaction by alcoholic alkali solution. It was considered as methyl glyoxal-bis-2,4-dinitrophenylhydrazone. It was ascertained by paper partition chromatography. Yellow precipitates were mainly osazones of acetaldehyde. Pyruvic acid was also identified from urine.

The reddish precipitates were solved in one or two drops of pyridine, and by paper chromatography methyl glyoxal was identified at the original spot ($R_f = 0$). Of course this would not exclude simple glyoxal, phenyl glyoxal, and diacetyl, but Prof. H. Tatsuta[5] in Organic Chemistry of our University succeeded at last in separating these substances at his own work by suitable solvent (70 cc. acetone +30 cc. water including 1 cc. petroleum ether) and ascertained that the spot of the bottom ($R_f = 0$) was methyl glyoxal itself highly probable. I was thus able *to identify methyl glyoxal not only from human milk, but from human urine.* Details will be published in this Journal.

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On the Swelling of Erythrocytes in Isotonic Solution of Various Cations in Cases of Several Internal Diseases

By

Hiroshi Takahagi

(高 萩 浩)

(From the Medical Clinic of Prof. F. Nakazawa, Faculty of Medicine, Tohoku University, Sendai)

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When a pathological mechanism occurs in a human body, many remarkable abnormal phenomena, namely symptoms come into sight and at the same time some delicate changes can be seen directly or indirectly. Taking the phenomenon of swelling of erythrocytes under a certain condition, the problem has been investigated in cases of various diseases.

With regard to the swelling of tissue and substance Loeb[1] and others[2)-7)] have made fundamental investigations of the human tissue and especially the muscle, gelatine and some glue board. In our laboratory Sato,[8)] Oyama and Sone,[9)] Shimizu and Takahagi[10)] have made experiments on the swelling of some tissues. The research on the swelling of erythrocytes which play the greatest role in keeping the vital activity of human body and reacting quickly upon the pathological mechanism, have been done by Koeppe[11)] and others.[12)-15)] However these studies were only basic and theoretical and no investigation has been made directly on patient's blood. To this date, the true mechanism of swelling is still unclassified. Hofmeister[2)] considered it as a nonchemical phenomenon between the swelling body and the medium: Loeb and others considered that the swelling in a body was due to a gelatinaus compound of protein and water; Schwarz,[17)] Spiro and Ostwald[18)] and others have proved experimentally and theoretically that pH, salts, and the other conditions cause complicated effects to the gelatine compound to induce a complicated reaction; but, whatever be the case, the swelling is merely to be considered that the volumé of the swelling body increases as the result of absorbing the surrounding water in itself. Needles to say, the swelling body in an organism is protein. It is a self-evident truth that a medium including protein absorbs water into it and is influenced by quantitative and qualitative change of that protein. Oyama and Sone[9)] in our laboratory proved that there is a parallel relation between the degree of the swelling of tissue

and the colloid-osmotic pressure when the tissue protein is changed into a condition of sol, so therefore, as erythrocytes consists of a great deal of protein, it can be stated that the major cause for the swelling of erythrocyte is protein in the erythrocytes.

Nakazawa[19] has previously reported that protein and colloid-osmotic pressure of blood plasma show various changes in various diseases. Therefore I believe that protein in erythrocytes may disclose some changes in various grades in various diseases and that the swelling of erythrocytes under a certain condition will be able to show minute variations. As there are many complicated factors in a human body, the phenomenon of swelling may become very complicated; however, we can experiment on the swelling in vitro in constant condition very easily. It is my hope that this work will give some convenience to the diagnosis and judgment of prognosis of diseases, by undertaking the study on the swelling of erythrocytes in various diseases under the above mentioned condition.

Experimental

Method

As medium solution I used the isotonic chloride solutions of 4 kinds of cations: Na, K, Ca and Mg (0.9% NaCl, 1.14% KCl, 2.52% $CaCl_2$ and 2.34% $MgCl_2$). Most of the examines were the inpatients of Prof. Nakazawa's Medical Clinic. Blood samples were taken from patient's cubital vein free from congestion prior to the patient's breakfast time, and immediately after defibrination, centrifuged in an electric centrifuge (3000 R.P.M.) for 30 minutes, mixed thoroughly after removing the blood serum, a layer of leucocytes and a small amount of erythrocytes. 4 test-tubes each containing 2.5 cc. of above mentioned isotonic cation solutions were prepared and to which 0.5 cc. of erythrocytes was added respectively. A homogenous suspensive fluid of erythrocytes was thereby obtained by gently mixing the solutions. The test-tubes were then allowed to stand 30 minutes in an incubator at 37°C. The measuring of the swelling was undertaken immediately after removing the test-tubes from the incubator. In order to measure the swelling, I used a small graduated tube for precipitation which was espécially made for this purpose: a glass-tube 100 mm. long, with a true inside diameter of 5 mm., graduation of 150 and 1.5 cc. volume.

Examination procedure was as follows: 1.5 cc. of the mixture of erythrocytes and cation solution was poured into each of the 4 tubes for precipitation, and centrifuged at 3.000 R.P.M. for 20 minutes. Then, noted the volume of swelling by reading the height of the layer of erythrocytes at the bottom of the tubes. As an aiding factor, the rest of mixture of erythrocytes and cation solutions were placed in Westergren's

tubes for blood sedimentation rate, and read the sedimentation rate every hour for 6 hours while being kept in a box whose temperature was constantly kept at 20°C. However, by Westergren's method, the results cannot be clearly observed because of the hemolysis which occurs in the course of observation, therefore Westergren's method is suitable for reference purposes only. For sake of convenience, the swelling in the Na solution is represented by Na-swelling and likewise: K-swelling represents the swelling in K-solution. Ca-swelling represents the swelling in Ca-solution. etc.

Results

1. Healthy persons

26 persons were selected for experiment—12 males and 14 females—from a group of healthy laboratory workers, laboratory assistants and nurses. These persons were examined and their swelling of erythrocytes was measured as shown in Tab. I.

The values of swelling by this method, under average conditions, were: 2.0–2.7 on Na-swelling, 2.0–2.6 on K-swelling, 2.0–2.5 on Mg-swelling and 1.4–2.0 on Ca-swelling. Ca-swelling showed the least visual change. It was difficult to read the values of the swelling of other solution by Table observations only. Statistical examination was made as 26-persons by use of various cations as indicated in Fig. 1: On comparing Na-swelling with K-swelling, no significant difference was found: the level of significance is 5%, because the mean of the difference of individual values (M) is +0.042 and the unbasised estimate of population variance (U^2) is 0.052.

When comparing K-swelling with Mg-swelling, $M=+0.173$, and $U^2=0.045$, were obtained. In such a case the significant difference, though very small, existed and the fact was recognized that Mg-swelling is less than K-swelling. In the next instance, comparing Mg-swelling with C-swelling, $M=+0.519$ and $U^2=0.093$, were obtained and thereby showing a remarkable and a significant difference. The value of Ca-swelling was very small compared with that of Mg-swelling, i.e., the fact was established that Ca-swelling was the least of the 4 kinds of cations, and accordingly, the order of the value of swelling of erythrocytes in each of the cation solution was: $Na=K>Mg>Ca$.

According to the former study on the swelling of a piece of liver by Sato,[8] Oyama and Sone,[9] and Shimizu and Takahagi[10] in our laboratory, the order of the values of swelling in Na, K, Mg and Ca, for swelling of liver tissue was found to be as follows: $K>Na>Ca>Mg$. This order has a quite different appearance as compared with that of erythrocytes, and especially it showed a remarkable difference i.e., Ca-swelling was less than

TABLE I

Nr.	Age and gender	NaCl	KCl	$MgCl_2$	$CaCl_2$
1	26, ♂	2.5	2.5	2.1	2.0
2	30, ♂	2.5	2.35	2.0	2.0
3	31, ♂	2.3	2.3	2.3	1.8
4	41, ♂	2.35	2.8	2.75	2.0
5	18, ♂	2.3	2.25	2.15	1.7
6	20, ♂	2.4	2.35	2.1	1.05
7	14, ♂	2.4	2.3	2.5	1.2
8	20, ♂	2.0	2.05	2.15	1.7
9	19, ♂	2.4	2.4	2.3	1.4
10	42, ♂	2.6	2.1	2.4	1.9
11	32, ♂	2.4	2.5	2.3	1.7
12	60, ♂	1.8	2.0	2.1	1.6
13	22, ♀	2.7	2.4	2.5	1.9
14	21, ♀	2.4	2.35	2.0	2.0
15	21, ♀	2.5	2.4	2.25	1.4
16	20, ♀	2.5	2.4	2.5	2.0
17	53, ♀	2.8	2.5	2.8	2.3
18	30, ♀	2.7	2.0	2.35	1.7
19	22, ♀	2.8	2.6	2.4	2.1
20	20, ♀	2.5	2.4	2.15	1.7
21	18, ♀	2.15	2.4	2.0	1.6
22	19, ♀	2.3	2,5	1.75	1.7
23	19, ♀	2.5	2.45	2.1	1.9
24	27, ♀	2.6	2.6	2.6	2.0
25	23, ♀	2.4	2.5	2.1	1.6
26	25, ♀	2.4	2.5	2.3	1.8

Mg-swelling. It is recognized the fact that there is a peculiarity pertaining to the swelling of erythrocytes.

2. Tuberculous diseases

The advancement of tuberculous diseases and the erythrocyte sedimentation rate are closely related to each other as proved by numerous scholars, such as: Westergren[20] and after him Benninghof[21] and others.[22]-[33] These writers have reported that the cause of above fact is due to the increase of globulin and fibrinogen in blood. Ito, Seki and Nakazawa[34] have proved that the colloid-osmotic pressure of the blood of phthisical patients is generally within the range of normal values among the light cases and decreases in serious cases. Referring to the reports on this problem and from our own observations it is concluded that some change must have occurred in its colloid-chemical state, that is, the quality

Fig. 1

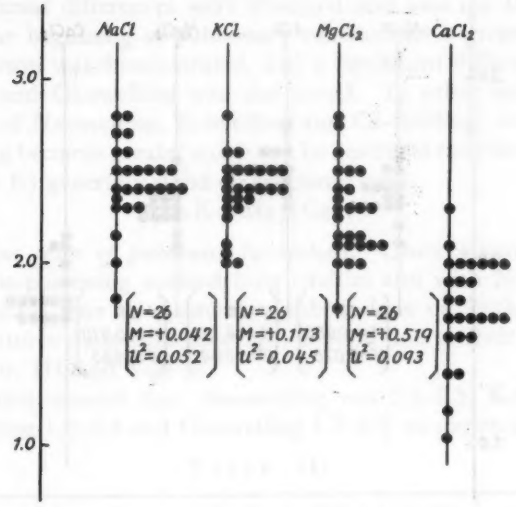
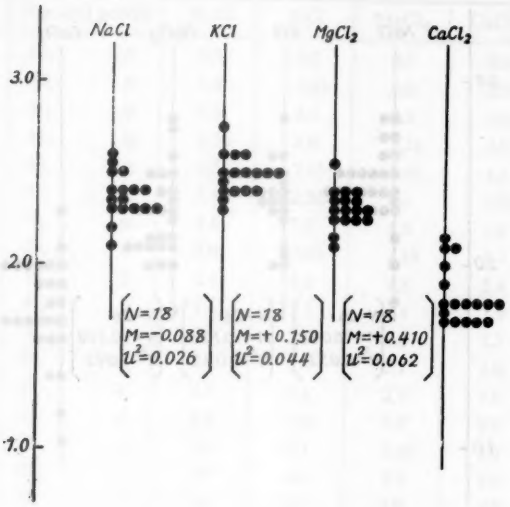


TABLE II

Nr.	Age and gender	NaCl	KCl	$MgCl_2$	$CaCl_2$
1	20, ♂	2.1	2.4	2.25	1.9
2	16, ♂	2.5	2.5	2.4	2.15
3	26, ♂	2.3	2.4	2.1	1.8
4	28, ♂	2.4	2.4	2.35	1.8
5	22, ♂	2.3	2.5	2.35	2.1
6	24, ♂	2.35	2.5	2.3	1.7
7	21, ♂	2.3	2.5	2.25	2.0
8	59, ♀	2.2	2.3	2.3	1.7
9	26, ♀	2.3	2.35	2.15	1.7
10	20, ♀	2.3	2.4	2.35	2.1
11	19, ♀	2.35	2.6	2.4	1.8
12	24, ♀	2.55	2.6	2.4	1.8
13	18, ♀	2.4	2.5	2.55	1.8
14	19, ♀	2.5	2.6	2.3	1.7
15	32, ♀	2.4	2.45	2.25	1.8
16	28, ♀	2.4	2.5	2.3	1.75
17	31, ♀	2.6	2.75	2.25	1.7

of bloodprotein must have changed in cases of tuberculous diseases. With regard to the quantitative change of mineral matters in blood in cases of tuberculous diseases, Otto[85] and others[86]-[40] have made numerous re-

Fig. 2.



ports during their days. Some variations have been found in their reports, though scarcely no scholar has denoted any special important variations. Close observation of qualitative change of bloodprotein which is closely connected with swelling, discloses that there are some qualitative changes in the erythrocyte colloid, and therefore, I undertook the problem of tuberculous diseases and its relation to the swelling of tubercular patients erythrocytes.

Tubercular patient were divided into 3 groups; i.e. 1) slight cases, 2) serious cases of pulmonary tuberculosis, 3) tuberculous pleurisy, peritonitis and meningitis.

i) Slight cases of pulmonary tuberculosis. Observation on 17 slight cases of pulmonary tuberculosis resulted in data as stated in Tab. II. These patients appeared to have slight symptoms as clearly indicated by clinical diagnosis and X-ray examinations. The patient's swelling results were: Na-swelling 2.3–2.5; K-swelling 2.4–2.6; Mg-swelling 2.2–2.4 and Ca-swelling 1.7–2.0. At a glance, we can not recognize the great difference comparing with healthy persons on the volume and the order of the each swelling. Statistic figures as in Fig. 2, show that a slight difference can be detected. No significant difference exists between Na-swelling and K-swelling as in healthy persons, and there is no significant difference between K-swelling and Mg-swelling, because $M=+0.150$, $U^2=0.044$, as found when observing healthy persons. When the value of Mg-swelling

of slight pulmonary patients was compared with healthy persons statistically, minute significant differences were observed and also the fact that Mg-swelling at the beginning of pulmonary tuberculosis is greater than that of healthy person was demonstrated, and a significant difference between Mg-swelling and Ca-swelling was also noted. In other words, there is little change of Na-swelling, K-swelling and Ca-swelling: only the value of Mg-swelling becomes greater and it can be described that there 3 swellings (Mg, Na and K) generally stand on the same level.

$$Na = K = Mg > Ca.$$

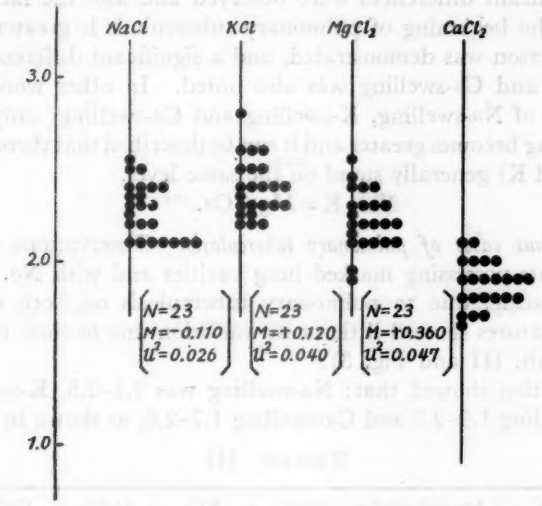
ii) *Serious cases of pulmonary tuberculosis.* Observations were made on 23 patients possessing marked lung cavities and with No. 4 or more of Gaffky's scale, due to pulmonary tuberculosis on both side, whose body temperatures showed little or no variation due to their being in the hospital. (Tab. III and Fig. 3).

Observation showed that: Na-swelling was 2.1–2.5, K-swelling 2.2–2.6, Mg-swelling 1.9–2.5 and Ca-swelling 1.7–2.0, as shown in the Table.

TABLE III

Nr.	Age and gender	NaCl	KCl	$MgCl_2$	$CaCl_2$
1	21, ♂	2.4	2.6	1.9	1.8
2	28, ♂	2.4	2.3	2.1	1.9
3	33, ♂	2.1	2.2	1.95	2.0
4	70, ♂	2.35	2.5	2.3	2.1
5	27, ♂	2.3	2.35	2.1	2.0
6	27, ♂	2.2	2.4	2.1	1.9
7	38, ♂	2.3	2.25	2.2	1.8
8	19, ♂	2.1	2.4	2.2	1.9
9	29, ♂	2.25	2.4	2.45	2.0
10	32, ♂	2.1	2.3	2.2	1.9
11	27, ♀	2.1	2.8	2.1	1.8
12	22, ♀	2.1	2.25	2.1	2.1
13	22, ♀	2.3	2.4	2.4	1.9
14	27, ♀	2.2	2.3	2.2	2.0
15	24, ♀	2.5	2.4	2.3	1.7
16	23, ♀	2.4	2.3	2.15	1.65
17	32, ♀	2.1	2.2	2.2	1.9
18	50, ♀	2.2	2.3	2.4	1.9
19	32, ♀	2.1	2.2	2.3	1.8
20	25, ♀	2.55	2.6	2.4	1.8
21	18, ♀	2.4	2.5	2.55	1.8
22	18, ♀	2.5	2.6	2.3	1.7
23	21, ♀	2.35	2.5	2.25	1.7

Fig. 3.



Comparing these results, M=—0.110, U^2=0.026 were obtained between Na-swelling and K-swelling; this fact is quite opposited to cases in healthy persons who show M=+0.042. Next, comparing K-swelling with Mg-swelling, M=+0.120, U^2=0.040 were obtained. In comparison with that of healthy persons Mg-swelling value approximates that of K-swelling. That is, Mg-swelling shows a greater comparative value, Ca-swelling is always the least and shows scarcely any change. In other words, Na-swelling tends to be lesser, whereas Mg-swelling a little larger in the serious cases of pulmonary tuberculosis.

$$Na < K = Mg > Ca$$

iii) *Tuberculous pleurisy, peritonitis and meningitis.* Observations were made on 23 cases suffering from tubercular pleurisy, peritonitis and tuberculous meningitis. The results are shown in Tab. IV and Fig. 4. As shown in the table, Na-swelling was 2.1–2.5, K-swelling 2.3–2.6, Mg-swelling 1.8–2.4 and Ca-swelling 1.7–2.5. It was observed by comparing these results that M=0.100, U^2=0.010 between Na-swelling and K-swelling. This fact is opposite to the cases of healthy persons from whom M=+0.042 was obtained, and the contraction of Na-swelling as well as in the serious cases of pulmonary tuberculosis was recognized. Comparing K-swelling with Mg-swelling, M=+0.170 and U^2=0.044 were obtained; Mg-swelling approximates K-swelling when compared with the cases of healthy persons—this fact shows that Mg-swelling is augmenting. Ca-

TABLE IV

Nr.	Age and gender		NaCl	KCl	$MgCl_2$	$CaCl_2$
1	17,	♂	2.3	3.3	1.75	2.5
2	19,	♂	2.1	2.2	2.3	1.55
3	35,	♂	2.15	2.3	2.1	1.6
4	22,	♂	2.1	2.3	2.2	1.4
5	22,	♀	2.5	2.4	2.5	2.0
6	21,	♀	2.1	2.3	2.4	2.5
7	23,	♀	2.5	2.6	1.9	2.6
8	26,	♀	2.55	2.6	2.4	1.8
9	22,	♀	2.6	2.8	2.55	1.8
10	21,	♀	2.35	2.5	2.1	2.4
11	21,	♀	2.2	2.25	2.5	1.9
12	23,	♀	2.1	2.3	2.2	1.8
13	29,	♀	2.1	2.3	2.0	1.4
14	59,	♀	2.2	2.3	2.3	1.7
15	18,	♀	2.2	2.15	1.8	1.4
16	32,	♀	2.4	2.6	2.4	1.7
17	41,	♀	2.2	2.3	1.7	1.55
18	23,	♀	2.4	2.3	1.8	1.65
19	23,	♀	2.25	2.45	2.3	2.1
20	19,	♀	2.35	2.6	2.35	1.95
21	27,	♀	2.1	2.3	2.05	1.8
22	25,	♀	2.55	2.6	2.4	1.8
23	19,	♀	2.6	2.6	2.6	1.9

swelling is always the least and shows scarcely any changes compared with that of healthy persons. As stated above, the order of the swelling is the same as during the serious cases of pulmonary tuberculosis. It is obvious that the lesser value of Na-swelling, the greater is the increasing of the value of Mg-swelling.

$$Na < K = Mg > Ca$$

The above mentioned measurements were made on such patients who had entered the critical stages of their illness, and therefore, in order to examine the recovery stage, 13 patients were selected for observation, those who are in the convalescent stage as based on the findings of clinical examination. The results are shown in Tab. V and Fig. 5, which show: Na-swelling 2.1–2.4; K-swelling 2.2–2.4; Mg-swelling 2.0–2.3; and Ca-swelling 1.7–1.9. Comparing these results, $M = -0.031$, $U^2 = 0.007$ were obtained between Na-swelling and K-swelling; the significant difference which have existed among the same group of patients at their critical stages disappears, and the value of Na-swelling approximates that of K-

Fig. 4.

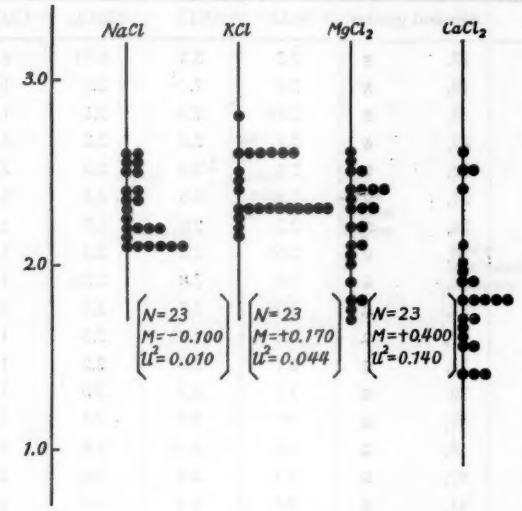
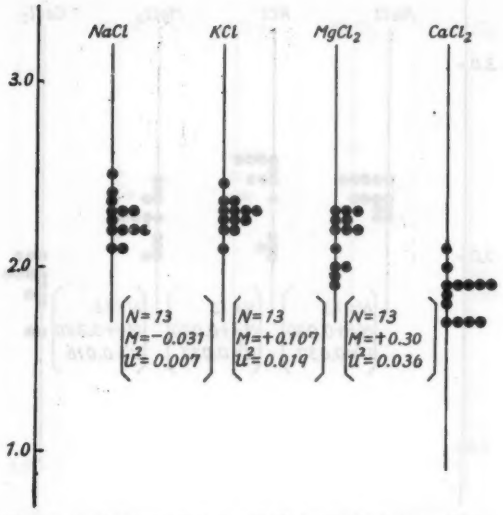


TABLE V

Nr.	Age and gender	NaCl	KCl	$MgCl_2$	$CaCl_2$
1	17, ♂	2.35	2.3	2.2	2.1
2	41, ♂	2.2	2.25	1.9	1.9
3	19, ♂	2.1	2.1	2.0	1.7
4	30, ♂	2.1	2.2	2.25	1.9
5	28, ♂	2.2	2.25	2.2	1.85
6	37, ♀	2.4	2.3	2.3	2.0
7	21, ♀	2.3	2.2	1.95	1.9
8	29, ♀	2.2	2.25	2.2	1.8
9	19, ♀	2.25	2.3	2.0	1.9
10	18, ♀	2.2	2.3	2.1	1.7
11	27, ♀	2.3	2.35	2.25	1.9
12	32, ♀	2.3	2.35	2.3	1.7
13	27, ♀	2.5	2.45	2.3	1.7

swelling and the fact that the order of swelling approximates that of healthy persons was established. This fact will also be found when comparative examinations on the Na-swelling of healthy persons is made statistically. Comparing K-swelling with Mg-swelling, $M=+0.107$, $U^2=0.019$ which show less significant difference than in healthy persons. Between Mg-swelling and Ca-swelling there is a relationship that $M=+0.300$, $U^2=$

Fig. 5.



0.036, and significant difference is recognized in this case. Observations confirmed the fact that the order of erythrocytes swelling comes near to that of healthy person. That is: Na=K>Mg>Ca.

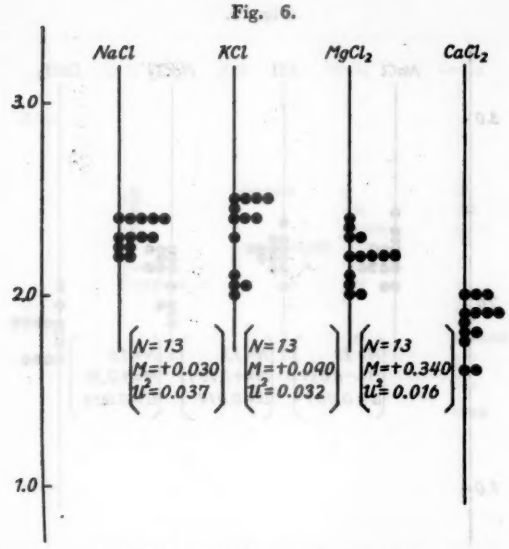
3. Cardiovascular diseases

The swelling measurements were made on 13 patients (11 patients with valvular diseases and 2 patients with aortic aneurysm) and the results are shown in Tab. IV and Fig. 6. Na-swelling is 2.2–2.4, K-swelling

TABLE VI

Nr.	Age and gender	NaCl	KCl	$MgCl_2$	$CaCl_2$
1	35, ♂	2.25	2.3	2.35	1.85
2	52, ♂	2.4	2.5	2.3	2.0
3	50, ♂	2.4	2.4	2.3	1.9
4	51, ♂	2.3	2.4	2.2	1.6
5	39, ♂	2.25	2.1	2.0	1.6
6	22, ♂	2.4	2.5	2.2	2.0
7	19, ♂	2.2	2.05	2.1	1.8
8	46, ♂	2.2	2.4	2.2	1.9
9	47, ♀	2.4	2.5	2.4	1.9
10	40, ♀	2.3	2.5	2.2	1.9
11	57, ♀	2.4	2.45	2.2	2.0
12	25, ♀	2.3	2.05	2.0	1.75
13	24, ♀	2.3	2.0	2.05	1.8

Fig. 6.



2.1–2.5, Mg-swelling 2.0–2.3 and Ca-swelling 1.6–2.0. By statistical investigation, it was found that $M=+0.030$, $U^2=0.037$ between Na-swelling and K-swelling and no significant difference existed. Next, $M=+0.090$, $U^2=0.033$ were obtained between K-swelling and Mg-swelling. Mg-swelling is rather small than K-swelling, and $M=+0.34$, $U^2=0.016$ were obtained between Mg-swelling and Ca-swelling. These values show that Ca-swelling too is very small. This relationship is similar to the case of healthy persons and the order is:

$$N=K>Mg>Ca.$$

There is no remarkable change in this cases. In addition, as shown in Table VII and Fig. 7, 6 cases of essential hypertension were examined, but they can not be objects of the accuracy of the statistical examination.

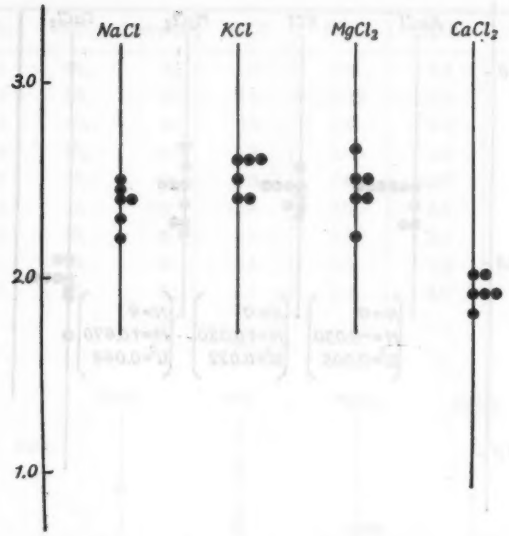
4. Nephritis

It is well known fact that in cases of nephritis there is always some

TABLE VII

Nr.	Age and gender		NaCl	KCl	$MgCl_2$	$CaCl_2$
1	57,	♂	2.4	2.5	2.65	1.9
2	53,	♂	2.3	2.4	2.2	1.8
3	56,	♂	2.4	2.6	2.5	1.9
4	62,	♂	2.2	2.4	2.4	2.0
5	56,	♀	2.45	2.6	2.5	1.9
6	46,	♀	2.5	2.6	2.4	2.0

Fig. 7.

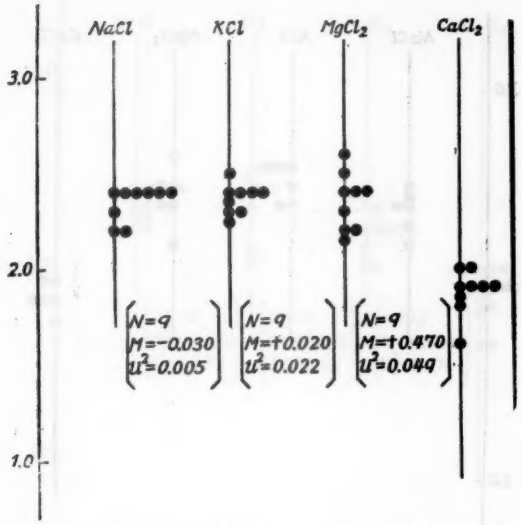


variations of the colloid contained in the human tissue. Therefore, as it can be expected that there is some variation in the protein of erythrocyte, the swelling measurements were made on 9 nephritis patients, and as shown in Tab. VIII and Fig. 8, which show Na-swelling 2.2–2.4, K-swelling 2.3–2.4, Mg-swelling 2.2–2.4 and Ca-swelling 1.9–2.0. As sufficient patients for observation in this particular case were not available and also difficult to express statistically and theoretically, however, as shown in Fig. 8, no remarkable difference existed between Na-swelling and K-swelling, and it is also difficult to recognize the significant difference between

TABLE VIII

Nr.	Age ond gender	NaCl	KCl	$MgCl_2$	$CaCl_2$
1	44, ♂	2.4	2.4	2.5	2.0
2	29, ♂	2.2	2.3	2.4	1.8
3	30, ♂	2.4	2.4	2.6	1.6
4	40, ♂	2.4	2.4	2.3	1.9
5	51, ♂	2.2	2.25	2.2	1.9
6	50, ♂	2.4	2.35	2.4	2.0
7	55, ♀	2.4	2.5	2.2	1.9
8	19, ♀	2.4	2.3	2.15	1.85
9	32, ♀	2.3	2.4	2.4	1.9

Fig. 8.



K-swelling and Mg-swelling. But by calculation, the difference between K-swelling and Mg-swelling, $M=+0.020$, $U^2=0.022$ were obtained and both were regarded as being nearly equal. A remarkable difference existed between Mg-swelling and Ca-swelling. Here, Ca-swelling is always the least, and the order of swelling is as follows:

$$Na=K=Mg>Ca.$$

In nephritis, Mg-swelling increases as compared with that of a healthy person.

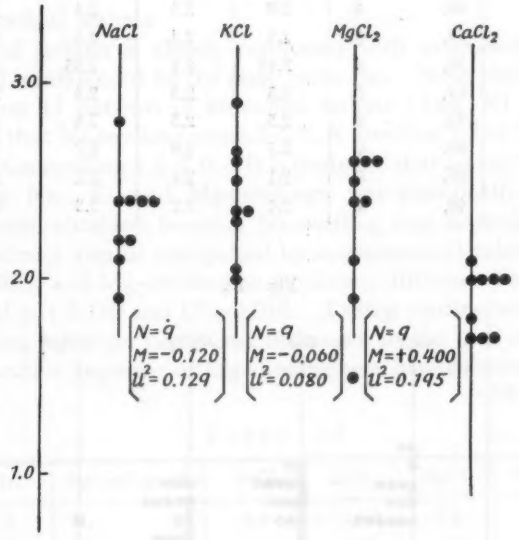
5. Anemia

Observations were undertaken mainly on 9 patients with simple anemia, and as shown in Tab. IX and Fig. 9 which show: Na-swelling 2.0–2.4, K-swelling 2.0–2.5, Mg-swelling 2.0–2.6 and Ca-swelling 1.7–2.0. The value of Ca-swelling showed a slight decrease whereas Mg-swelling an increase. In this case, a statistical examination in the strict sense of the term can not be conducted, but, when experimented on patients, calculation was tried on the assumption that it is possible to do so. It was found that $M=-0.120$, $U^2=0.124$ between Na-swelling and K-swelling mean a minute contraction of Na-swelling. There is a relation that $M=-0.06$, $U^2=0.080$ between K-swelling and Mg-swelling, and any difference is not to be recognized. It is obvious that there is a comparative augment of Mg-swelling in such a case. Here, it is needless to say that Ca-swelling is the least, and therefore the order of swelling is

TABLE IX

Nr.	Age and gender	NaCl	KCl	$MgCl_2$	$CaCl_2$
1	48, ♂	2.2	2.5	2.4	1.8
2	58, ♂	2.4	2.35	2.5	1.7
3	20, ♂	2.1	2.0	2.1	1.7
4	19, ♂	1.9	2.65	1.5	2.0
5	51, ♀	2.8	2.6	2.6	2.1
6	31, ♀	2.4	2.05	2.6	1.7
7	16, ♀	2.2	2.35	2.4	2.0
8	16, ♀	2.4	2.3	1.9	2.0
9	28, ♀	2.4	2.9	2.6	2.0

Fig. 9.



supposed to be as follows:

$$Na < K = Mg > Ca.$$

The comparative augment of Mg-swelling and contraction of Na-swelling will be recognized in a case of noticeable anemia.

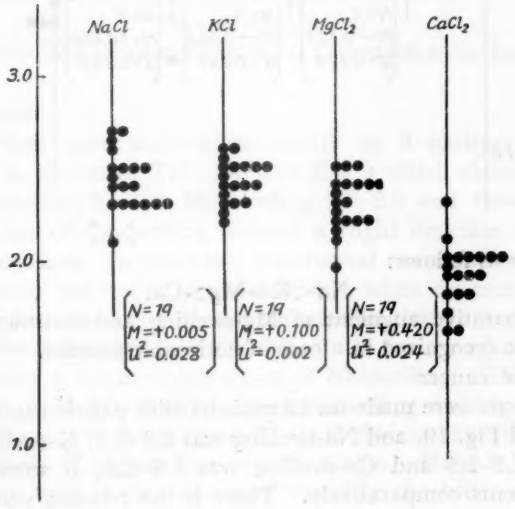
6. Gastric cancer

Observations were made on 19 patients with gastric cancer as shown in Tab. X and Fig. 10, and Na-swelling was 2.3–2.5, K-swelling 2.3–2.5, Mg-swelling 2.2–2.5 and Ca-swelling was 1.6–2.0; it seems that Mg-swelling augments comparatively. There is the relation which is: M=

TABLE X

Nr.	Age and gender		NaCl	KCl	$MgCl_2$	$CaCl_2$
1	52,	♂	2.5	2.4	2.5	1.6
2	49,	♂	2.7	2.5	2.1	1.8
3	56,	♂	2.65	2.3	2.2	1.9
4	36,	♂	2.3	2.4	2.15	1.95
5	56,	♂	2.3	2.5	2.3	2.0
6	53,	♂	2.4	2.45	2.25	1.7
7	62,	♂	2.5	2.55	1.95	2.3
8	25,	♂	2.3	2.25	2.2	1.8
9	62,	♂	2.4	2.6	2.5	2.0
10	29,	♂	2.3	2.35	2.4	1.9
11	55,	♂	2.3	2.3	2.5	2.1
12	66,	♂	2.6	2.5	2.4	1.9
13	63,	♂	2.5	2.3	2.4	2.0
14	38,	♂	2.45	2.4	2.35	1.9
15	62,	♂	2.3	2.5	2.4	2.0
16	49,	♂	2.5	2.5	2.4	1.6
17	46,	♀	2.7	2.6	2.2	1.8
18	66,	♀	2.1	2.4	2.2	2.0
19	49,	♀	2.4	2.2	2.3	2.0

Fig. 10.



+0.005, U^2=0.028 between Na-swelling and K-swelling. Here, of course, no significant difference exists, but U^2 and M are small, compared with a healthy person. This fact proves that the value of Na-swelling is nearly equal to that of K-swelling, i.e. a slight decrease of Na-swelling. Next, K-swelling was compared with Mg-swelling, and so the results were M=+0.100, U^2=0.002. And there was no significant difference between K-swelling and Mg-swelling; and therefore comparative augument of Mg-swelling was proved. There was a very remarkable difference between Mg-swelling and Ca-swelling, the latter was the least. Therefore the order of swelling can be stated as:

$$Na \fallingdotseq K = Mg > Ca.$$

It was considered that there is a comparative augment of Mg-swelling and that comparative contraction of Na-swelling was probable in the cases of gastric cancer.

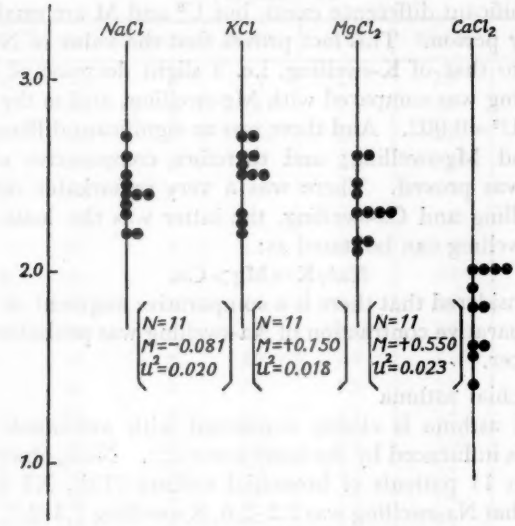
7. Bronchial asthma

Bronchial asthma is closely connected with autonomic nerves and blood colloid is influenced by the same nerve too. Now, observations were undertaken on 11 patients of bronchial asthma (Tab. XI and Fig. 11). It was found that Na-swelling was 2.2–2.6, K-swelling 2.3–2.7, Mg-swelling 2.3–2.6 and Ca-swelling 1.6–2.0. It is probable that no marked difference exists among Na-, K- and Mg-swellings, but statistically M=—0.081, U^2=0.020 were obtained between Na-swelling and K-swelling, therefore a slight difference can be recognized by mathematical calculations. Between K-swelling and Mg-swelling no significant difference can be observed because of M=+0.150 and U^2=0.018. Taking consideration of decrease of Mg-swelling with the significant difference in the case of healthy persons, comparative augment of Mg-swelling was demonstrated in this case.

TABLE XI

Nr.	Age and gender	NaCl	KCl	$MgCl_2$	$CaCl_2$
1	21, ♂	2.4	2.5	2.3	1.9
2	24, ♂	2.5	2.55	2.45	2.0
3	31, ♂	2.6	2.7	2.6	1.8
4	20, ♂	2.2	2.5	2.3	1.8
5	25, ♂	2.2	2.3	2.15	1.6
6	31, ♀	2.45	2.7	2.4	2.0
7	26, ♀	2.4	2.25	2.1	1.4
8	23, ♀	2.35	2.2	2.15	1.6
9	20, ♀	2.7	2.6	2.6	2.0
10	59, ♀	2.3	2.5	2.3	1.55
11	19, ♀	2.4	2.6	2.3	2.0

Fig. 11.



Ca-swelling is the least, therefore the order of swelling can be stated as:

$$Na \fallingdotseq K = Mg > Ca.$$

It is supposed that there is an augment of Mg-swelling and a little comparative decrease of Na-swelling in bronchial asthma.

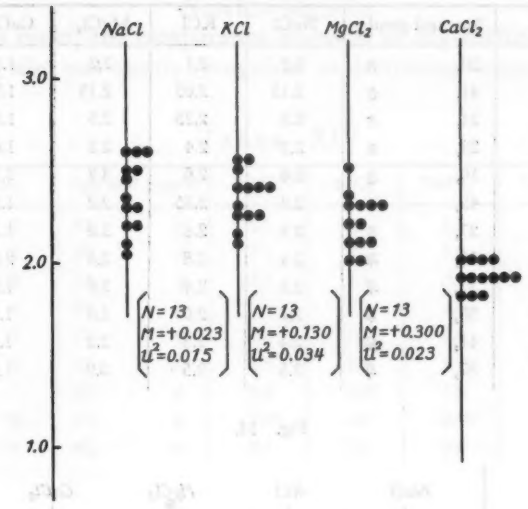
8. Beriberi

Observations were made on 13 patients with beriberi as shown in

TABLE XII

Nr.	Age and gender	NaCl	KCl	$MgCl_2$	$CaCl_2$
1	22, ♂	2.3	2.4	2.0	1.8
2	14, ♂	2.6	2.55	2.3	1.8
3	22, ♀	2.6	2.4	2.5	1.9
4	28, ♀	2.05	2.1	2.1	1.9
5	18, ♀	2.2	2.25	2.1	2.0
6	24, ♀	2.5	2.55	2.3	1.9
7	22, ♀	2.45	2.4	2.35	2.0
8	29, ♀	2.1	2.15	2.2	1.9
9	26, ♀	2.6	2.5	2.0	1.9
10	41, ♀	2.3	2.25	2.3	2.0
11	23, ♀	2.2	2.25	2.1	1.9
12	22, ♀	2.5	2.3	2.3	2.0
13	21, ♀	2.35	2.4	2.2	1.8

Fig. 12.



Tab. XII and Fig. 12. As generally accepted, the present-day beriberi-patients showed only slight cardiovascular and sensory disturbance with or without traces of edema. The results of this experiments is as follows: Na-swelling is 2.2–2.6, K-swelling 2.2–2.5, Mg-swelling 2.1–2.3 and Ca-swelling 1.8–2.0, and the order is the same as of healthy persons. Examination by calculation disclosed that $M=+0.023$, $U^2=0.015$ between Na-swelling and K-swelling. There is not so much significant difference as in the case of healthy persons, and where $M=+0.130$, $U^2=0.034$ were obtained between K-swelling and Mg-swelling. The value approximates that of healthy persons. Between Mg-swelling and Ca-swelling $M=+0.030$, $U^2=0.023$ were obtained which showed a remarkable difference. Therefore, at least, in the cases of beriberi as observed the variation on the order of swelling can not be recognized and therefore:

$$Na=K>Mg>Ca.$$

9. Croupous pneumonia and typhoid fever

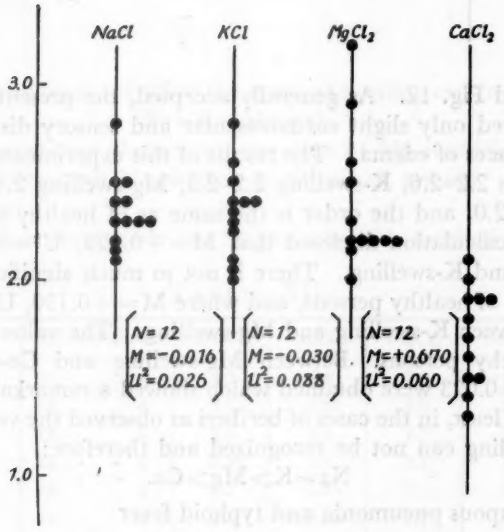
Observations were made on 12 patients with acute croupous pneumonia and 9 patients with typhoid fever (Tab. XIII and Fig. 13, Tab. XIV and Fig. 14).

Firstly, pneumonia patients were observed and Na-swelling was 2.2–2.5, K-swelling 2.1–2.5, Mg-swelling 2.2–2.5 and Ca-swelling as 1.5–1.9. By comparing these results between Na-swelling and K-swelling, $M=-0.016$, $U^2=0.023$ were obtained and there was no significant difference,

TABLE XIII

Nr.	Age and gender		NaCl	KCl	$MgCl_2$	$CaCl_2$
1	20,	♂	2.2	2.1	2.2	1.5
2	41,	♂	2.15	2.05	2.15	1.3
3	21,	♂	2.3	2.25	2.5	1.8
4	23,	♂	2.3	2.4	2.2	1.6
5	10,	♂	2.8	2.6	3.2	2.1
6	49,	♂	2.3	2.35	2.2	1.9
7	37,	♂	2.3	2.4	2.2	1.7
8	56,	♀	2.4	2.8	2.3	2.0
9	37,	♀	2.5	2.4	2.6	1.9
10	38,	♀	2.1	2.0	2.0	1.4
11	41,	♀	2.4	2.3	2.2	1.5
12	37,	♀	2.5	2.5	2.9	1.9

Fig. 13.



but, in effect it is a probable case that there is a comparative decrease of Na-swelling compared with healthy person. Between K-swelling and Mg-swelling, $M=-0.03$, $U^2=0.088$, which showed no significant statistical difference. Comparative augment of Mg-swelling was found the same as healthy persons. There is a remarkable difference between Mg-swelling and Ca-swelling statistically, which is rather greater than in the case of

healthy person, and expressed as:

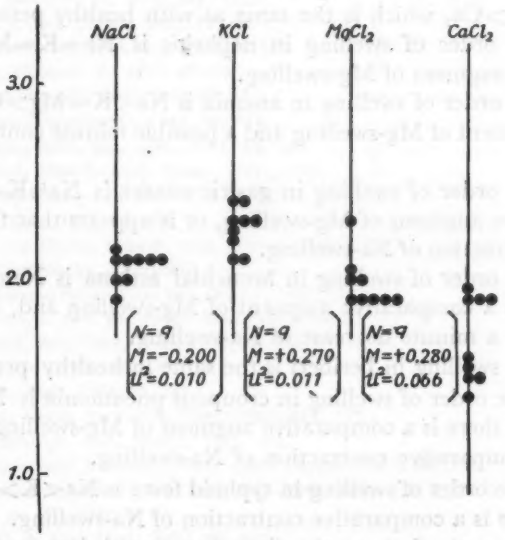
$$Na \leqq K = Mg > Ca.$$

By this order, the comparative augment of Mg-swelling and that of Na-swelling also was clearly recognized as decreasing comparatively.

TABLE XIV

Nr.	Age and gender	NaCl	KCl	$CaCl_2$	$MgCl_2$
1	32, ♂	2.1	2.1	1.9	1.55
2	30, ♂	2.1	2.2	1.9	1.6
3	43, ♂	2.1	2.4	2.3	1.5
4	44, ♂	2.1	2.3	2.0	1.95
5	22, ♂	2.0	2.3	1.9	1.9
6	37, ♀	1.9	2.1	1.9	1.4
7	23, ♀	2.0	2.25	1.9	1.5
8	17, ♀	2.15	2.4	2.05	1.9
9	41, ♀	2.1	2.3	2.0	1.9

Fig. 14.



Typhoid fever patients showed the order of swelling as per Tab. XIV as also as follows: $Na < K > Mg > Ca$. Which shows that the decrease of Na-swelling is a considered fact.

Summary

By summarizing the facts and data obtained from the fore-mentioned experiments:

1. The order of erythrocytes swelling in healthy person is

$$Na = K > Mg > Ca$$

and being different from Hofmeister's order, and is proper only to erythrocyte.

2. On tuberculous disease, it has been demonstrated that there is a comparative augment of Mg-swelling in the beginning of pulmonary tuberculosis, and a comparative contraction of Na-swelling and comparative augment of Mg-swelling in the serious cases of pulmonary tuberculosis. By these facts, if Na-swelling turns to normal, it may be considered that it indicates an improvement of the malady.

3. In tuberculous pleurisy, peritonitis and meningitis, the comparative contraction of Na-swelling and the comparative augment of Mg-swelling was recognized in the critical stage of the illness, but in the patients of tuberculous pleurisy and peritonitis who are on their way to recovery, this relationship turns to normal.

4. The order of swelling in cardiovascular disease is recognized as $Na = K > Mg > Ca$, which is the same as with healthy persons.

5. The order of swelling in nephritis is $Na = K = Mg > Ca$, or a comparative augment of Mg-swelling.

6. The order of swelling in anemia is $Na < K = Mg > Ca$, or a comparative augment of Mg-swelling and a possible minute contraction of Na-swelling.

7. The order of swelling in gastric cancer is $Na \fallingdotseq K = Mg > Ca$, or a comparative augment of Mg-swelling, or it appears that there is a comparative contraction of Na-swelling.

8. The order of swelling in bronchial asthma is $Na \fallingdotseq K = Mg > Ca$. There is also a comparative augment of Mg-swelling and, as a matter of fact, there is a minute decrease of Na-swelling.

9. The swelling in beriberi is the same in healthy persons.

10. The order of swelling in croupous pneumonia is $Na \fallingdotseq K = Mg > Ca$. Besides there is a comparative augment of Mg-swelling, and possibly there is a comparative contraction of Na-swelling.

11. The order of swelling in typhoid fever is $Na < K > Mg > Ca$, and possibly there is a comparative contraction of Na-swelling.

To sum up the facts on the above mentioned data in a few words as possible, the swelling of erythrocytes in the case of Na, K, Mg and Ca—4 kinds of cation, have a peculiarity, i.e., having a different appearance against the swelling of tissue of liver or others. In the case of disease, the

order of swelling is varied, but the variations in the cases of K-swelling and Ca-swelling are very small: it is Mg-swelling that causes the main variation, and is Na-swelling is a secondary swelling to a Mg-swelling.

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F. Nakazawa.

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A Method of Collecting the Renal Vein Blood for the Study of the Liberation of Renin in Non-anesthetized Animals

By

Tatuzi Suzuki and **Toshiyuki Ozaki**
(鈴木 達二) (尾崎 俊行)

(From the Department of Physiology, Nagasaki University School of Medicine, Nagasaki)

(Received for publication, December 17, 1951)

Our method is an application of the method of Satake, Sugawara and Watanabe,[1] which enabled us to collect the suprarenal vein blood specimens without anesthesia, laparotomy, fastening or evoking any pain, to the sampling of the renal vein blood specimens.

For the study of the liberation of renin, Bouckaert and De Landsheere[2] devised a method to obtain specimens of renal vein blood in anesthetized dog in which interferences with the blood circulation are reduced to minimum. However by using their method it is not easy to perform experiments in non-anesthetized animals.

The procedure of our method is as follows. In dogs or cats, the section of D_{11}-L_3 dorsal spinal roots was performed previously under anesthesia, as in the method of Satake *et al.* After some weeks, the left renal vein is exposed through the lumbar route without anesthesia. The left spermatic vein, which joins in the most of cases to the left renal vein, is prepared and doubly ligated at a distance of about 1.5 cm. from the opening of the vein and cut off between the two ligatures. Near the opening, the vein is clamped and a small cannula for collecting the samples is inserted just below the clamp. Then a thick silk thread is passed round the renal vein just near the entrance. To collect the blood samples the silk thread round the vein is gently pulled and the clamp on the spermatic vein is removed.

The usefulness of this method in dogs could be ascertained by us in some experiments.

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A Roentgen-ray Figure, Characteristic to the Diverticulum of the Stomach

By

Rai Satake
(佐 武 萊)

(From the Medical Clinic of Prof. T. Kurokawa, Faculty of Medicine, Tohoku University, Sendai)

(Received for publication, July 27, 1951)

In a patient, showing a roentgen figure like the gastric diverticulum, some trials were immediately followed with success by placing him in a proper position to obtain the figures, wholly characteristic to that developmental anomaly. They are really the mucous reliefs, not only in the neck of diverticulum but also in the diverticulum sac itself. While the former was emphatically mentioned by Beutel and Mahler[1] and Berg[2] twenty years ago, there is no previous reference of the latter.

Fig. 1 on the next page indicates both reliefs clearly.

As I came across, as above stated, the roentgen picture like the diverticulum in the cardiac region on the side of lesser curvature, I let the patient lay down for a while in the supine position. Ample time was given for completely filling the sac therewith. Then let him turn to the prone position, but inclined 45 degrees with the head downwards, something like the Trendelenburg position, but prone. The position was changed in repetition once more. And finally a photograph was taken in the erect right oblique position.

No further comment is needed for Fig. 1.

That these two roentgen figures are essential to fix the diagnosis as the diverticulum, implys, otherwise expressed, that most of the figure, taken by previous investigators as characteristics to the gastric diverticulum, cannot be taken nowadays as such, as will be discussed in followings:

Firstly the air bubble[1, 3-8] at the top of sac is also often seen in the Haudek form of ulcer niche.[9, 10]

Fig. 2 can be taken as a good demonstration of the true diverticulum, developed at the cardia on the lesser curvature. In spite of repeated examinations such a roentgenogram was never obtainable, while a little cascade stomach was finally discovered.

By way of precaution it may be noted: A roentgenogram, almost

Fig. 1

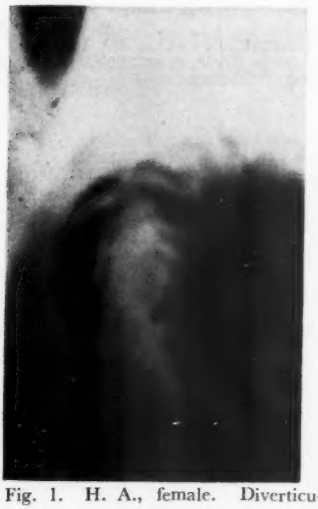

Fig. 1. H. A., female. Diverticulum of the stomach. Mucous reliefs in the neck and sac.

Fig. 2

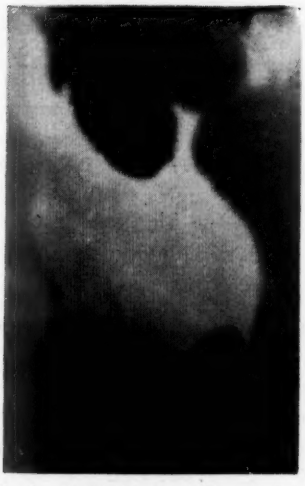

Fig. 2. H. H., female. Diverticulum like figure (upper middle); in fact a little cascade stomach.

Fig. 3

Fig. 3. I. A., female. Cardiac cancer (middle).

the same to Fig. 3 was diagnosed by a writer[11] as the diverticulum. The figure, however, must be taken at once, as a matter of fact, as the cardiac cancer, and this case was verified as so at the operation.

Secondly, Åkerlund[8] and others[1, 6-8, 11, 12, 15, 16] laid stress upon the finding that the contrast medium stays 1–24 hours in the diverticulum, what was quite recently seconded by Brown *et al.*[13] The same figure is to be reasonably expected for a suspended ulcer niche, located near the cardia.

In fact, I had an opportunity to observe a residue of the contrast medium, remaining in a penetrating ulcer niche, as large as a bean, 28 hours after the barium administration. It is readily comprehensible if it will be realized that in such a case as above described the muscular layer is already interrupted, so that no peristalsis propagates therein. Consequently the contrast medium can be evacuated more slowly than in the true diverticulum, where the muscular apparatus remains wholly intact. Further as the diverticulum is smooth in contour, as referred to by Åkerlund[8] and others,[1, 6-8, 12] the mucous layer can be legitimately taken also as smooth, in contrast to ulcer niche, which effects also the evacuation of barium, to be sure.

Thirdly, the mucous reliefs streaks, converging toward the orifice of diverticulum can not be taken as characteristic to the diverticulum as mentioned by Berg.[2] The figure can be observable in the ulcer niche too.

Lastly, the peristalsis is doubtless an important proof for the diverticulum, but it might be very difficult or almost impossible to get it, if it be situated in the cardiac region. The peristalsis takes place in that region most weakly, so that Holmes and Robbins[11] are of opinion that normal peristalsis sets in at about the middle of corpus when studied roentgenologically. The more important the polygraphical study rê the matter, the more desirable the publication of graphs. (Cf. 15, 16.)

Some words will be now spent about the studies on the position of patients in roentgenography in similar conditions to the present. Berg[2] intended to fill the sac with contrast medium completely there. Roux and Béclère[17] were able to get a good X-ray photography of the cardiac diverticulum by letting a patient stand on the head by use of a special elaborate apparatus of de Gaiffe.

In order to see the relief figure, it is however necessary to convert the position, once the head down, then up, as above emphasized.

As appendix the frequency of occurrence of the gastric diverticulum, may be mentioned. A case report of Kanomata and Shirahata,[16] with rather complete previous references rê the matter, home and overseas, was the first case in our clinique, and the present case the 6. In 10 years

and in fact among the gastric patients of ca. 20,000, we had also 6 cases, i.e. 0.03%, what harmonizes with those reported in U.S.A. etc.[5, 7, 18]

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Usefulness of the Supine and Prone Position on a Plane Inclinable 45 Degrees for Fluoroscopy of Digestive Tract

By

Rai Satake
(佐武　薬)

(From the Medical Clinic of Prof. T. Kurokawa, Faculty of Medicine, Tohoku University, Sendai)

(Received for publication, July 27, 1951)

In the foregoing paper[1] I am able to show how readily a good relief of a diverticulum in the cardiac region is obtainable only by changing the position in certain manners; namely by letting the subject lay on the head alternatively supine and prone on a plane inclined 45 degrees. The contrast medium fills thus once the sulci in the mucosa of cardia of one side and then those of the opposite side.

This procedure, though quite simple in principle and manner, has been proved very useful for the roentgenography as following examples will demonstrate.

(1) Corpulent persons has commonly horn-shaped stomach, situated in the upper epigastrium, or further up under the costal arch. Large tumour mass in the abdominal cavity, ascites, the late stadium in pregnancy, etc. push the stomach high up: In such a case the mucous relief cannot be obtainable by means of the compression method of Berg[2].

My method can be readily applied there:

Fig. 1a is a roentgenogram of a corpulent patient, diagnosed firstly as the stomach cancer, but there is no mucous relief visible. As seen in Fig. 1b, constructed from Fig. 1 and three other roentgenograms, the tumour mass, a child head-sized, is found in the place of the pancreas head, and surrounded by the duodenal loop, so that the so-called duodenal fenestra is formed. On that account it was taken as the pancreas cancer infiltrating into the stomach tissue.

On treating by my procedure, and in fact in the second, left anterior oblique position, a clear full ring figure showing a dish-shaped cancer was uncovered in the antrum, near the pylorus. (Fig. 1c) Irregularities of the mucous relief and their abrupt discontinuation were demonstrated there. The operation findings corresponded to the latter roentgenogram; in lymph glands in the mesenterial radix there were found some metastasis,

Fig. 1a

Fig. 1a. K. I., male. Stomach cancer, no mucous reliefs visible (in prone position); Compare Fig. 1c.

Fig. 1b

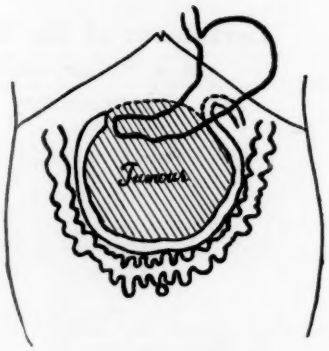

Fig. 1b. The same patient as Fig. 1a.

Fig. 1c

Fig. 1c. The same patient as Fig. 1a. The full ring figure about in the middle, further see the text.

forming a tumour of a child head size. The pancreas remained entirely unaffected.

(2) Our method is also applicable to cancers of stomach, even when the tumour mass is too large for the spoon distinctor or when it is not touched on screen palpation.

Fig. 2 shows the mucous relief figure from a cancer, which was too large so that only the half mass was made clear by the use of distinctor.

Fig. 3a, demonstrating the full ring figure, was obtained on the left oblique position, practised according to our method, while the screen palpation method did not yield any sign of tumour on direct palpation.

Fig. 2

Fig. 2. F. T., female. Stomach cancer. About all the figure shows the cancer; the left, duodenum; the right, intact portion of stomach.

Fig. 3b, indicating the half ring figure, was taken after having letting the patient stand erectly. It is the cancer, developed in the antrum. This was operated with the results well corresponded to these figures.

(3) The coecum mobile. Commonly it is diagnosed by measuring the position of its lower end in the figures taken at the erect position and at the supine and those taken when the coecum pushed asides manually right and left.

Fig. 3a

Fig. 3a. K. K., male. Stomach cancer. The full ring figure in the left; normal reliefs in the right.

Fig. 3b

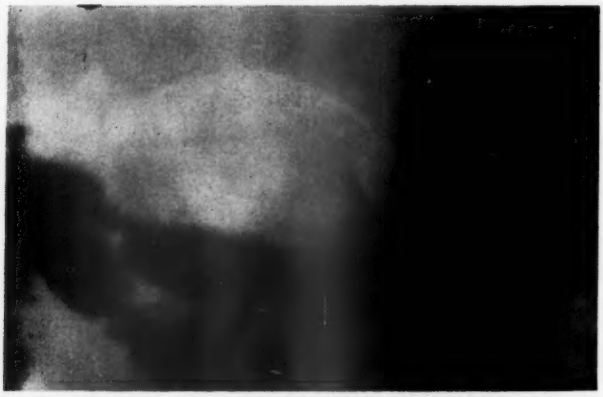

Fig. 3b. The same patient. The half ring figure, no mucous reliefs visible.

It is here quite obvious that the position supine with the head down, on the plane inclined 45 degrees, more or less, as above repeating given, distinctly exaggerate the difference. Fig. 4 demonstrates this.

Fig. 4.

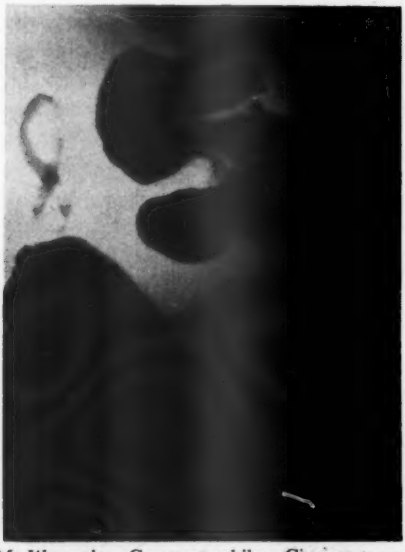

Fig. 4. M. W., male. Coecum mobile. Coecum turns over, and the appendix thereabove; on the right side colon ascendens.

The coecum turns towards cranial, and the appendix thereabove.

The movil elongated S-romanum can also likewise easily photographed.

Further our method is also applicable for diagnosing female pelvic organs[3] and also for determining the upper limit of cancer infiltration even when the oesophagus tumour is not accompanied by a stricture. A similar idea was related to by Palugyay[4] previously of roentgen examination of the oesophagus.

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A Convenient Type of Chest Electrode for Human Electrocardiography

By

Shigenori Kameyama
(亀山 重徳)

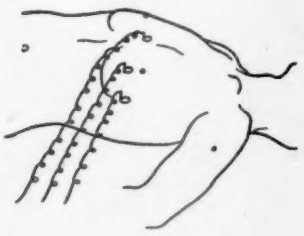
(From the Laboratory of Applied Physiology, Faculty of Medicine, Tohoku University, Sendai. Director: Prof. K. Matsuda)

(Received for publication, January 24, 1952)

Needless to say, the precordial leads are nowadays the routine in clinical electrocardiography. While several types of special chest electrode have been devised so far, they are not sufficiently convenient in use. The electrode here described is very simple, and can be applied easily and quickly anywhere on the body surface, without any accessaries such as rubber bandage, suction cup etc.

It is a small disc, cut circular from a thin plate of the same metal as the limb electrodes, e.g., German silver (thickness about 0.2 mm.). On one side of it is soldered an enameled, thin copper wire (B. S. # 37) which is led to the amplifier appropriately. The electrode, being very light, can be pasted on the body surface with ordinary salted paste, and never slips down even when applied on the vertical side of the body (Fig. 1). Application, removal and reapplication can always be done easily and quickly. Until now, I took the precordial leads ECG with this electrode (diameter 1.5 cm.) several hundred times and encountered no difficulties at all.

Fig. 1



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On the Rôle of the Augmented Adrenaline Secretion from the Suprarenal Glands after Inhalation of Carbon Monoxide upon the Fluctuation of the Blood Sugar Content and the Blood Pressure Simultaneously Occurring*

By

Tatuzi Suzuki, Kingo Takahasi and **Kahei Tamabuti**
(鈴木 達二) (高橋 謹吾) (玉渕 嘉平)

(From the Physiological Laboratory of Prof. Y. Satake, Tohoku University, Sendai)

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The occurrence of remarkable hyperglycemia in carbon monoxide poisoning is the well known fact.[1)-16)] Nevertheless concerning the part played by the suprarenal medulla in the carbon monoxide hyperglycemia, sufficient information is lacking.

According to Starkenstein[1)] the carbon monoxide hyperglycemia was not found in rabbits after the removal of suprarenals. On the other hand Bornstein and Holm[3)] detected the remarkable carbon monoxide hyperglycemia in both kinds of rabbits, normal and suprarenalectomized. In two suprarenalectomized rabbits the carbon monoxide experiments were carried out by Yanagawa.[14)] The hyperglycemia of a small scale was observed in one rabbit, while in the other the diminution of blood sugar content was found.

It is generally accepted that after carbon monoxide inhalation the blood pressure elevates at first and then descends.[17)-22)] But contrary to this general view, Yano and Kirita[6)] observed non-influence of carbon monoxide inhalation upon the blood pressure in rabbit. The relation of the adrenaline secretion from the suprarenal medulla to the fluctuation of blood pressure produced by carbon monoxide inhalation was not hitherto known.

The augmentation of adrenaline secretion after carbon monoxide inhalation, which was suggested by some experimentalists, was proved and precisely determined by Wada *et al.*[22)] in our laboratory.

With the desire to elucidate the share of the augmented adrenaline

* Reported at the XXV Annual Meeting of the Japanese Physiological Society at Niigata 1948, May.

secretion of the suprarenal medulla after the carbon monoxide inhalation in the hyperglycemia and the blood pressure elevation thereby occurring, the present investigation was undertaken.

Experimental

Method

The experiments were carried out in the following manner: in the first place carbon monoxide was inhaled in the normal dogs, secondly the same in the dogs, deprived of the suprarenal medulla, and lastly in the dogs, so operated on, the adrenaline solution was injected intravenously with the velocity imitating the natural adrenaline secretion from the suprarenal medulla in normal dog after carbon monoxide inhalation.

The demedullation of the suprarenals was carried out several days or weeks before the experiment through the lumbar route under ether anesthesia by the two stage operation. The medullary tissue was spooned out and cauterized with a red hot tongs.

All the experiments were carried out without narcotizing or fastening. The blood sugar content and the blood pressure was estimated in all series of experiments. The blood specimens for the blood sugar estimation were collected from a branch of the external jugular vein and the estimation was carried out by the method of Hagedorn and Jensen. The blood pressure was registered by a mercury manometer from the femoral artery.

Carbon monoxide was made by dropping the formic acid into the heated concentrated sulphuric acid, and just before use the carbon monoxide was mixed with the air in 3%. The inhalation of the 3% carbon monoxide gas was performed through a glass cannula inserted into the trachea.

Adrenaline solution for continuous infusion was prepared by diluting adrenaline hydrochloride solution of Sankyo Co. with 0.9% NaCl solution. Before the dilution actual concentration of the adrenaline solution was ascertained by Kodama's[23] method.

Result

The data are presented in Table I.

3% carbon monoxide gas was inhaled in 5 normal dogs with the body weight of 4.8–8.1 g. Soon after the start of inhalation the respiration became fast and deep. Then the animal became restless and respired slowly and gaspingly. The bradycardia took place. Then the convulsions set in. At this time the carbon monoxide inhalation was stopped.

Thereafter the respiration and the pulse returned gradually to its initial state. The body temperature fall of a small degree was observed.

Concerning the blood pressure, it began to ascend soon after the start of inhalation. It ascended further till the remarkable bradycardia took place and exceeded the initial height by 36 to 58 mm. Hg. It remained after the end of carbon monoxide inhalation at a high level for a while and then it descended very slowly. Some minutes or about half an hour after the carbon monoxide inhalation the blood pressure descended somewhat below the initial level and then it elevated gradually as to resume its initial height.

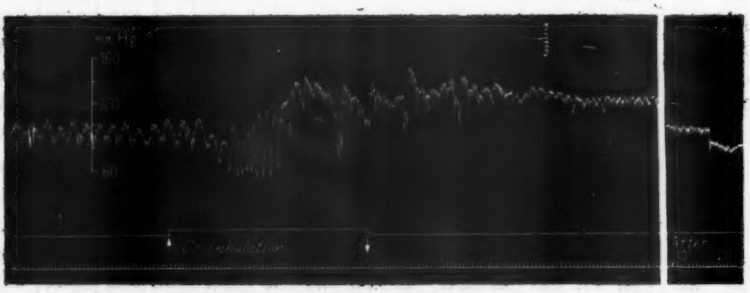

Fig. 1. Blood pressure tracings of Dog 4.
Normal dog, 6.7 kg. ♂, non-anesthetized. At arrow 3% carbon monoxide gas was inhaled. Time in seconds.

The blood sugar level was observed as already increasing 3 minutes after the start of carbon monoxide inhalation. The highest level was measured in the majority of cases 10 minutes after the inhalation. Then it decreased step by step. If the value obtained at each period of collection of blood specimens be averaged, its initial is determined as 0.105% and its maximum is found as 0.184%, which was observed 10 minutes after the inhalation. And the average duration of hyperglycemia was 90 minutes.

Quite the same experiments as in normal dogs were performed in 4 dogs with the body weight of 5.2–9.3 kg., whose suprarenal medulla was previously deprived of. Roughly speaking, the clinical symptoms in these dogs with the demedullated suprarenals after the carbon monoxide inhalation were almost similar with those in normal dogs. The fluctuation of blood pressure in these dogs after the carbon monoxide inhalation was almost the same as in normal dogs. The highest level exceeded the initial by 36–66 mm. Hg. It is difficult to find any notable difference in the magnitude of blood pressure elevation caused by the carbon monoxide anoxia between the normal dogs and those with the demedullated suprarenals.

TABLE

The Blood Sugar Content and the Blood Pressure after

No. of dog & sex	Body weight (kg.)	Date of experiment	Demedullation of suprarenals		Body temperature (°C.)		Before inhalation or infusion (min.)	
			R	L	Initial	After inhalation or infusion	20′	10′
(I) Carbon monoxide inhalation in normal dogs.								
1 ♀	4.8	29. XI. '43			37.5	35.5–37.1	0.115	0.112
2 ♀	5.3	6. XII. '43			38.6	37.0–37.9	0.097	0.100
3 ♀	8.1	20. XII. '43			39.4	38.3–39.1	0.110	0.110
4 ♂	6.7	15. I. '44			37.3	35.8–37.2	0.104	0.109
9 ♀	6.1	6. VI. '44			38.8	37.1–37.9	0.097	0.095
						Average	*0.105*	*0.105*
(II) Carbon monoxide inhalation in dogs with demedullated suprarenals.								
7 ♂	5.2	7. III. '44	1. XI. '43	4. XII. '43	38.0	36.5–38.1	0.134	0.132
8 ♂	9.8	10. III. '44	20. IX. '43	9. X. '43	39.2	38.5–39.1	0.119	0.124
10 ♂	9.3	10. VI. '44	4. V. '44	29. V. '44	38.4	37.4–37.9	0.081	0.072
6 ♂	6.7	19. VI. '44	27. IX. '43	20. X. '43	38.1	36.5–37.3	0.074	0.075
						Average	*0.102*	*0.101*
(III) Continous adrenaline infusion in dogs with the demedullated sup-								
5 ♂	7.5	24. I. '44	11. X. '43	25. X. '43	38.4	39.5–38.5	0.090	0.090
6 ♂	7.4	31. I. '44	27. IX. '43	20. X. '43	38.7	39.2–38.7	0.089	0.093
10 ♂	9.3	21. VI. '44	4. V. '44	29. V. '44	39.2	39.8–39.2	0.086	0.084
						Average	*0.088*	*0.089*

The fluctuation of the blood sugar content after the carbon monoxide inhalation in dogs with the demedullted suprarenals was similar to that, of normal dogs. Averaging the value obtained at each period of determination, the initial is estimated as 0.101% and the maximum is determined as 0.164%, which was observed 10 minutes after inhalation. It is somewhat inferior to the maximum value in normal dogs. Duration of hyperglycemia in mean was 60 minutes.

In the dogs with the demedullated suprarenals the fall of body temperature was observed in almost the same degree as that in normal dogs.

In the last series of experiments, continuous intravenous infusion of adrenaline was performed in three dogs (7.4–9.3 kg.) with the demedullated suprarenals in the velocity as near as the rate of adrenaline secretion actually caused by carbon monoxide inhaltion.

The pattern of the adrenaline infusion was constructed from the

I

Inhalation of Carbon Monoxide of Infusion of Adrenaline

Blood sugar content (%)									Blood pressure (mm. Hg)		
After inhalation or infusion (min.)									Initial	Maximum	Elevation
3′	5′	10′	20′	30′	45′	60′	90′	120′			
	0.210	0.214	0.199	0.182	0.165	0.147	0.123		90	143	53
0.108	0.124	0.182	0.181	0.163	0.118	0.137	0.083	0.085	93	130	37
0.133	0.119	0.111	0.102	0.118	0.111	0.116	0.119	0.119	90	146	56
0.173	0.163	0.198	0.177	0.175	0.175	0.155	0.117	0.081	90	126	36
	0.129	0.213	0.193	0.172	0.141	0.110	0.099	0.083	65	123	58
	0.149	*0.184*	*0.170*	*0.162*	*0.142*	*0.133*	*0.108*				
0.181	0.172	0.153	0.149	0.145	0.134	0.134	0.126	0.112	103	148	45
0.146	0.170	0.191	0.154	0.132	0.131	0.124	0.108	0.102	95	148	53
	0.108	0.211	0.200	0.120	0.075	0.063	0.061	0.063	86	152	66
	0.120	0.101	0.102	0.075	0.063	0.061	0.063	0.072	86	122	36
	0.142	*0.164*	*0.151*	*0.123*	*0.107*	*0.098*	*0.090*	*0.087*			
rarenals.											
	0.108	0.110	0.106	0.108	0.075	0.073	0.066		81	91	10
	0.121	0.126	0.107	0.108	0.083	0.077	0.093		108	138	30
	0.095	0.111	0.093	0.077	0.075	0.066	0.072		85	106	21
	0.108	*0.116*	*0.102*	*0.098*	*0.078*	*0.072*	*0.077*				

figures obtained by the experiments of Wada *et al.* as follow.

Injection of adrenaline per kg. of body weight; 1) 0.68γ in 1.0 cc. in 60 seconds, 2) 0.75γ in 0.5 cc. in 30 seconds, 3) 1.5γ in 0.5 cc. in 30 seconds, 4) 0.82γ in 0.5 cc. in 30 seconds, 5) 2.2γ in 3.0 cc. in 3 minutes, 6) 2.4γ in 4.0 cc. in 4 minutes.

The dogs were always quiet during and after the adrenaline infusion. The blood pressure elevated and reached its highest height 2 minutes after the start of infusion. It exceeded the initial height by 10–30 mm. Hg. The blood sugar concentration was increased by the adrenaline infusion. In the mean value, its initial is estimated as 0.089% and its maximum as 0.116%. Therefore the magnitude of hyperglycemia is far smaller than the carbon monoxide hyperglycemia in dogs with the demedullated suprarenals.

From the comparisons of these three groups of experiments, it is

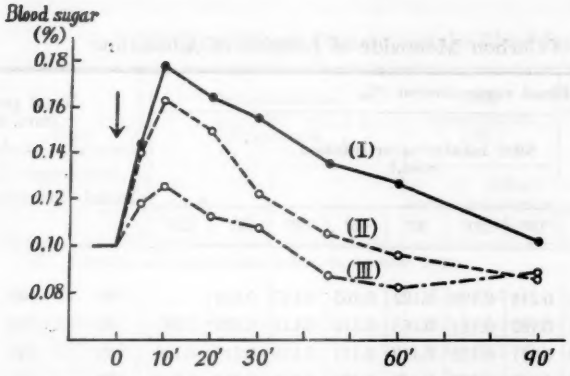

Fig. 2. Average blood sugar content in each series of experiments. Initial blood sugar content in each series is taken as 0.1%.
(I) Carbon monoxide inhalation in normal dogs.
(II) Carbon monoxide inhalation in dogs with the demedullated suprarenals.
(III) Continuous adrenaline infusion in dogs with the demedullated suprarenals.

readly known that the demedullation of the suprarenal gland reduces the magnitude of carbon monoxide hyperglycemia in a small degree, but it is never able to abolish the glycemic effect of carbon monoxide anoxia. The results of the previous authors, who could not observed the carbon monoxide hyperglycemia in suprarenalectomized animals, might be due to the lack of the cortico-adrenal function. It is justifiable to think that the carbon monoxide hyperglycemia is induced by two mechanisms: one is due to the augmented adrenaline secretion, and the other has no relation to the adrenaline secretion. The large part of the response is attributable to the latter mechanism.

Summary

To know what a part is played by accelerated adrenaline secretion in carbon monoxide anoxia in the fluctuation of blood sugar content and of blood pressure, the present investigations were undertaken.

Three series of experiments were carried out; (1) 3% carbon monoxide was inhaled in normal dogs. (2) The same was done in dogs with the demedullated suprarenals. (3) Continuous adrenaline infusion, which imitated the natural adrenaline secretion augmented by carbon monoxide was performed in dogs with the demedullated suprarenals. All experiments were carried out without narcotizing or fastening.

After inhalation of carbon monoxide, a remarkable hyperglycemia and elevation of the blood pressure were observed in normal dogs as well as in those with the demedullated suprarenals. The magnitude of hyper-

glycemia in dogs with the demedullated suprarenals was somewhat smaller than that in normal dogs. It was difficult to find any definite difference in the magnitude of blood pressure changes between the normal and the operated animals.

From the evidence given above it may be justifiable to conclude that the augmented adrenaline secretion from the suprarenal medulla plays a small part in the hyperglycemia produced by carbon monoxide poisoning.

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Mucosin and Mucoitin, Preliminary Note

By

Hajime Masamune, Zensaku Yosizawa and **Tyuiti Isikawa**

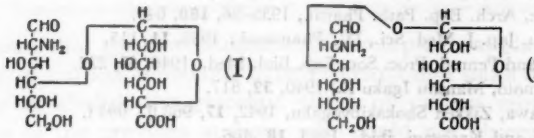
(正宗 一) (吉澤善作) (石川忠一)

(From the Medico-chemical Institute, Tohoku University, Sendai)

(Received for publication, February 3, 1952)

Investigaiing the structure of mucoitin (hyaluronic acid), Jeanloz[1] and Kurt H. Meyer[2] agreed with respect to the position in the glucuronic-acid-rests, wherewith acetylglucosamine links glucosidically, whereas the former author claimed the position in acetylglucosamine glucosidically bound by glucuronic acid to be 3–C and the latter 4–C. (The authors reasoned or assumed a pyranose structure of the monosaccharide components.)

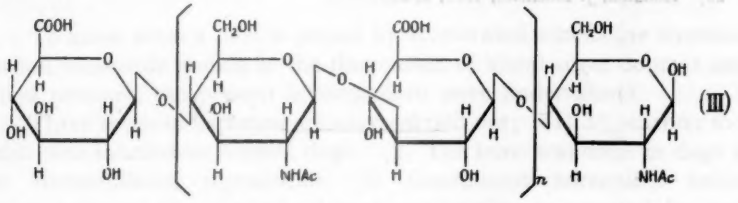
To begin with, the writers treated acetylmucosin-methylester (crystalline)[3] with periodic acid under the conditions preferred by Yosizawa.[4] The compound consumed 2.96 moles of the oxidant per mole, releasing 0.93 mole of formaldehyde (separated as the dimedon derivative),[5] which suggested (I) and (II) as possible structures of mucosin.



(I) (II)

β-Glucuronide structure has been established.[6]

Next pure mucoitin (from human umbilical cords; m.w. some 9300 from iodine use) was oxidized similar to above. It used 3 moles of the oxidant per mole, namely, the extremities of its chain reacted with the agent but not the internal part. Hence, a pyranose structure must be assigned to glucosamine units in mucoitin, because acetylglucosamine in the internal part of mucoitin, if it be a furanose, would use 1 mole of periodic acid per unit. Mucoitin is expressed in the formula below (III), if it is assumed that glucuronic acid units also possess a pyranose structure.



(III)

β-Glucosaminide structure is arbitrary.

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On the Biochemical Formation of the Free Vi Antigen, by the Resting or Acetone-killed Typhoid Organism

By

Isao Yamane

(山根 績)

(Department of Bacteriology, Faculty of Medicine, Tohoku University, Sendai. Director, Prof. M. Kuroya)

(Received for publication, August 20, 1951)

On preparing the typhoid vaccine, it has been considered as an indispensable property to preserve the Vi antigen in the vaccine.[1] But Vi antigen is very heat unstable and easily disappears from the bacterial bodies owing to cultural conditions.[2] Therefore to preserve Vi antigen in typhoid vaccine for a definite length of time is an important subject in this biological production. Nevertheless the studies on these aspects are very few and we can only find the studies of Gladstone[1] (1937) who researched the preserving conditions of Vi antigen in the synthic medium of typhoid bacilli.

So the author tested his results and assured his data again and in this studies found the fact that the cell free Vi antigen appeared in the culture filtrate on the pretty early stage of the incubation Further the author pursued the cell free Vi antigen, using the resting or acetone-killed cells of typhoid organisms and the present paper deals with these results.

Experimental

Materials and Method

Strains: *Salmonella typhosa* 58 (American strain from Panama Carrier) and *S. typhosa* 6S. Both keeping Vi antigen.

Suspension: The cells were washed twice with M/15[4] phosphate buffer of pH 7.4 and then suspended in the same buffer.

Shaking apparatus: Adjusted to 140 turns per minutes in frequency and 6 cm. in the shaking width and ordinarily incubated at 37 C.

Substrate: Glucose and other sugars were dissolved to 30 per cent in the above described phosphate buffer.

Titration: The supernatant of the shaken suspension was titrated by

means of precipitation test, using the anti-ballerup rabbit serum. The reading of the test was made after leaving for 5 hours at room temperature.

Results

Vi antigen formation by the resting cells in the presence of glucose and phosphate buffer

The cell suspensions of 58 or 6S strain were prepared to contain 50 mg. per 9 cc. phosphate buffer and were added to 1 cc. of 30 per cent glucose solution and poured into 100 cc. Erlenmeyer flask. The bottled suspensions were shaken at 37°C in the regular condition mentioned above. The supernatants of the cell suspension were titrated every one hour since the beginning of the incubation, so the formation of Vi antigen was traced in relation to time. As shown in Fig. 1, the precipitation titer increased

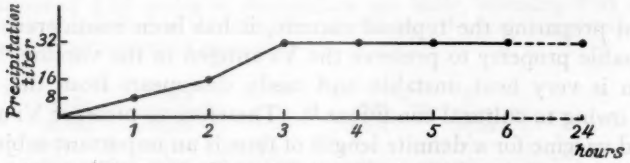
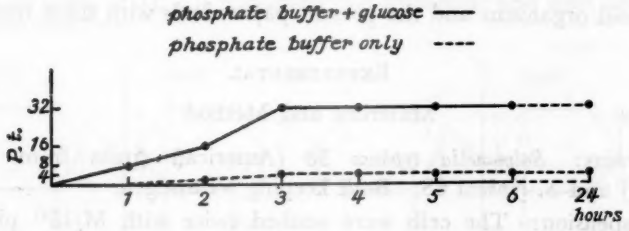


Fig. 1. The tracing of precipitation titer in relation to time.

to 32 times gradually till 3 hours of the incubation, but after then no increases were observed, showing that the equilibrium state was established since 3 hours. On the other hand in the case of the suspension containing no glucose, the titer after 3 hours incubation was 4 times and no increases were seen after then, as similarly as in the previous case. (Fig. 2) Further,

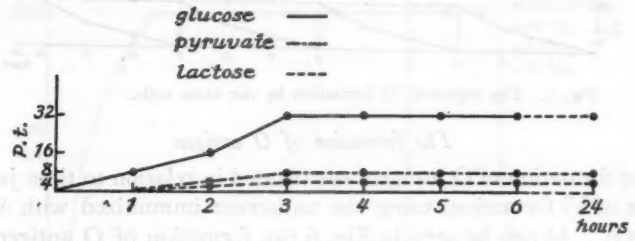
Fig. 2.



when the same concentration of lactate or pyruvate was added as a substitute for glucose, the formation of Vi antigen was very weak as can be seen in Fig. 3. When the saline or citrate[5] buffer suspension was used in place of phosphate buffer suspension, no Vi formation was observed.

The above described results were observed quite similarly both in the cases of 58 and 6S strain. When the same experiments were performed,

Fig. 3.



using the cells killed at 100°C for 1 hour, no Vi formation was observed. Nevertheless, in the case of the cells which were treated with acetone for 30 minutes and washed twice with phosphate buffer, the Vi formation were observed in the same manner as in the case of the resting cells. But when the treating time with acetone was over one hour, its activity was lost. So the cells treated with acetone for 30 minutes were used hereafter.

The difference of the activity using the subcultures of various incubating periods

The subcultures were harvested from 1 hour, 3 and 16 hours shaking culture. Fig. 4 shows that 3 hours cells were most active. But, the yield of 3 hours cells was very poor, so 16 hours cells were used hereafter.

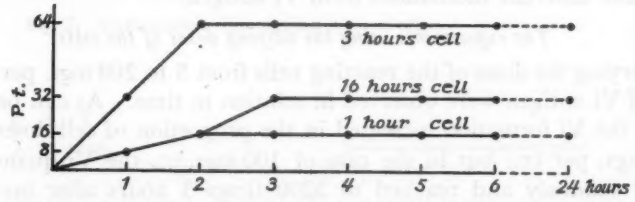


Fig. 4. The difference of the activity using the subcultures of various incubation periods.

The repeated Vi formation using the same cells

After shaking the cell suspension at the regular conditions for 3 hours, the supernatant was removed and the same amounts of phosphate buffer and glucose were added to the precipitated cells and the shaking was continued further, so the same treatment was repeated four times, using the same cells. In this case no decrease of activity was observed as can be seen in Fig. 5. and in this case the boiling supernatant of the precipitated cells after last treatment showed the same titer as were titrated before the incubation.

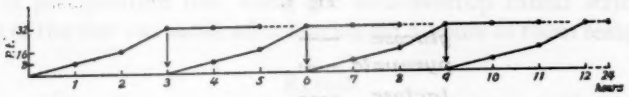


Fig. 5. The repeated Vi formation by the same cells.

The formation of O antigen

The formation of O antigen was titrated in relation to time just as in the case of Vi formation, using the antiserum immunized with *S. typhosa* 901 strain. As can be seen in Fig. 6 the formation of O antigen is very

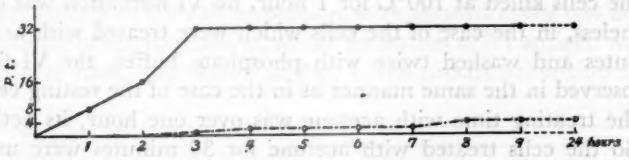


Fig. 6. Vi and O formation in relation to time Vi. ——, O —·—·

faint and its constant phase delayed for 2 or 3 hours, compared with the formation of Vi antigen. The formation of O antigen is always the same, even when glucose was replaced with lactate or pyruvate. The same results were obtained, using 901–0 strain which had only O antigen.

From above mentioned results, the O antigen appeared to be formed by a quite different mechanism from Vi antigen.

The experiments using the varying doses of the cells

Varying the doses of the reacting cells from 5 to 200 mgs. per cc., the titers of Vi antigen were observed in relation to time. As can be seen in Fig. 7, the Vi formation increased in the proportion of cell doses from 5 to 50 mgs. per cc., but in the case of 100 mgs./cc. the Vi quantities increased suddenly and reached to 3200 times 3 hours after incubation. Nevertheless in the case of 200 mg. per cc. there was no increase, compared with the case of 100 mg./cc.

The metabolism of the sugar and inorganic phosphorus in the medium

In order to assure whether the metabolism of sugar and inorganic phosphorus was performed, the amounts of the reducing sugar and inorganic phosphorus in the medium were estimated in relation to incubation time. Sugar estimation was performed according to Hagedorn-Jensen[6] while the inorganic phosphorus was estimated by Delory's method.[7][8]

As can be seen in Fig. 8, the reducing sugar decreased until 2 hours of incubation, but after then no decrease was observed, whereas the re-

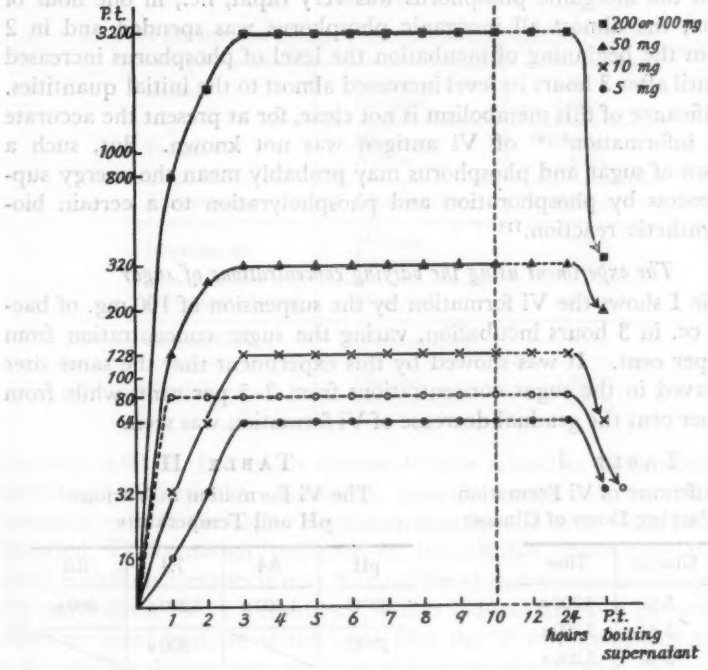


Fig. 7. Vi formation with varying doses of cells.

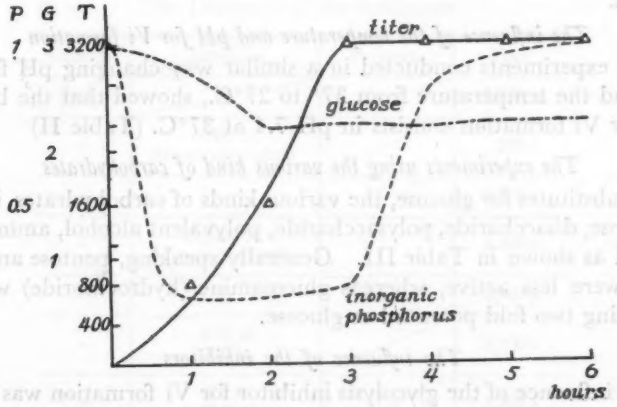


Fig. 8. The metabolism of glucose and inorganic phosphorus.

duction of the inorganic phosphorus was very rapid, i.e., in one hour of incubation, the almost all inorganic phosphorus was spended and in 2 hours from the beginning of incubation the level of phosphorus increased slowly, until after 3 hours its level increased almost to the initial quantities. The significance of this metabolism is not clear, for at present the accurate chemical information[9][10] of Vi antigen was not known. But, such a metabolism of sugar and phosphorus may probably mean the energy supplying process by phosphoration and phospholyration to a certain biological synthetic reaction.[11]

The experiment using the varying concentrations of sugar

Table I shows the Vi formation by the suspension of 100 mg. of bacteria per cc. in 3 hours incubation, varing the sugar concentration from 5 to 0.5 per cent. It was showed by this experiment that the same titer was observed in the sugar concentrations from 2–5 per cent, while from 1 to 0.5 per cent the gradual decrease of Vi formation was seen.

TABLE I

The Difference of Vi Formation with Varying Doses of Glucose

Glucose	Titer
5 %	3,200 x
3 %	3,200 x
2 %	3,200 x
1 %	1,600 x
0.5 %	800 x

Cell doses 100 mm./cc., incubation 3 hours.

TABLE II

The Vi Formation at Various pH and Temperature

pH	6.4	7.4	8.0
37°C	1,600 x	3,200 x	800 x
27°C		800 x	

Cell doses 100 mg./cc., incubation 3 hours.

The influence of the temperature and pH for Vi formation

The experiments conducted in a similar way changing pH from 6.4 to 8.0 and the temperature from 37° to 27°C., showed that the best condition for Vi formation consists in pH 7.4 at 37°C. (Table II)

The experiments using the various kind of carbohydrates

As substitutes for glucose, the various kinds of carbohydrates, i.e., pentose, hexose, disaccharide, polysaccharide, polyvalent alcohol, amino-sugar, was used as shown in Table III. Generally speaking, pentose and disaccharide were less active, whereas glucosamine (hydrochloride) was most active being two fold potential as glucose.

The influence of the inhibitors

The influence of the glycolysis inhibitor for Vi formation was studied. As the anaerobic inhibitor, 0.001 M. mono-iodoacetate[11] and as the aerobic

TABLE III

Vi Formation Using Various Kind of Carbohydrate

Hexose	Galactose	1,600x
	Levulose	3,200x
Pentose	Rhamnose	800x
	Xylose	800x
Amino-sugar	Glucosamine	6,400x
Disaccharide	Maltose	400x
	Lactose	400x
Polyvalent alcohol	Mannitol	1,600x
Polysaccharide	Gummi acasi	400x
Control	Glucose	3,200x
	P-Buffer only	400x

inhibitor, 0.05 M. Potassium cyanide[18] were added in its inhibiting concentration respectively. Potassium cyanide could not inhibit the Vi formation even in the higher doses than mentioned above, while iodoacetate inhibited Vi formation perfectly in its suitable concentration. From above mentioned results it may be considered that the anaerobic glycolysis will participate in Vi formation. When iodoacetate and pyruvate were added to basal medium at the same time the Vi formation was recovered to the similar degree with the case of glucose only. (Table IV)

TABLE IV

The Influence of the Inhibitors

Glucose	Inhibitor	Pyruvate	Titer
3%			3,200x
"	0.001 M Monoiodoacetate		100x
"	"	1.5%	3,200x
"	0.0005 M Monoiodoacetate		3,200x
"	"	1.5%	3,200x
"	0.05 M KCN		3,200x
"	0.1 M KCN		3,200x

Discussion

In 1902 Pick[14] reported that there appeared the antigen free from bacterial cells in the culture filtrate of typhoid bacilli. . Recently Hosoya[15] and his disciplines obtained the potential immunizing substances (named T.T.T. or T.A.T.) effective for typhoid like diseases from the culture filtrate of Salmonella families. But they did not research about the forming conditions of these substances in the broth.

On the other hand, in the group of the exotoxin producing bacteria its producing conditions were studied in details, and further the production of toxin was evident by shaking the resting cell suspension with addition of substrates, using *Streptococcus hemolyticus*,[16] *Shigella dysenteriae*,[17] *Clostridium tetani*,[18] and *Staphyloooooccus aureus*.[19] It will be an effective and appreciable method to use these methods in order to isolate the toxin in the relatively undenaturated and chemically definable state and to trace the metabolic path for the production of the specific substances. No research works on the latter subjects, however, are reported yet.

In the study reported here, it is necessary to take precautions against the possibility that the cell free Vi antigen may be liberated from the cells or eluted through the destruction of the cell bodies by the shaking treatment, for Vi antigen is considered to be in the outerlayer of the typhoid organism.[20] But from the view points, (1) that there was no decrease of the Vi activity even after the repeating of the shaking incubation four times with the same cells, and (2) that Vi formation was performed by employing the acetone-killed cells, and (3) that the glucose and inorganic phosphorus were necessary to be present and metabolized, accompaning the Vi formation, and (4) that there was an optimum concentration of sugar and the relative selectivity of sugar kind as the substrate, these phenomena appeared not to be due to the simple mechanical liberation from the cell, but may be due to the formation through a certain biochemical process. With regards to the formation process it may be considerable that the anaerobic glycolysis will participate in this reaction from the fact that it is not inhibited by potassium cyanide but by iodoacetate evidently. Further though it was inhibited by iodoacetate this inhibition was recovered immediately by adding the appropriate amounts of pyruvate, while pyruvate itself was not active for Vi formation. The iodoacetate can blockade the metabolic path from triose phosphate to phospho-glyceric acid and there is no energy donating process above the blockaded point. From above pointed results it may be presumed that triose phosphate or the other upper ordered metabolite of glycolysis will be used as the components of Vi substance and pyruvate may be available as the energy source of this reaction. Since the accurate chemical property of Vi, however, is quite

obscure now,[12)13)] so after purifying this cell free Vi and clarifying its chemical nature, this metabolic path would be able to be investigated further accurately.

Summary

1) When the acetone killed cell suspension of Vi-O and Vi-R type typhoid bacilli was shaken with glucose and phosphate solution at 37°C, the cell free Vi antigen was formed in the medium and this formation reached its maximum in 3 hours.

2) In spite of the repetition of the shaking incubations using the same cells, the Vi activity of the cells did not decrease. The cells metabolized glucose and inorganic phosphorus accompanying Vi formation.

3) The O-antigen formation by means of the same method was titrated very faintly at the later stage of the incubation. It was revealed that glucose and phosphate did not participate to O-formation.

4) Cell quantities, the optimum concentration of sugar and the suitable kind of the sugars were investigated.

5) Glucosamine was the most active in the Vi formation.

6) The Vi-formation was inhibited perfectly by iodoacetate and this inhibition was recovered immediately by adding pyruvate.

Sincere thanks are due to Prof. M. Kuroya for his kind guidance and revise.

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Pyruvic Acid and Acetaldehyde in Human Milk according to Different Arakawa's Reactions

By

Akira Sato and **Tsuneo Arakawa**
(佐藤 彰) (荒川 稚男)

(From the Department of Pediatrics, Faculty of Medicine, the Tohoku University, Sendai. Director: Prof. A. Sato)

(Received for publication, February 10, 1952)

This is an important remark upon Arakawa's two preceding Papers: "A Contribution[1] to the Method for the Quantitative Estimation of Pyruvic Acid in Human Milk" and "Pyruvic Acid content in Human Milk with Different Arakawa's Reaction."[2] As stated already, these works were completed in 1944. As recently shown by Wako's[3] preliminary paper, *methyl glyoxal* was identified by strict chemical method from Arakawa-negative milk and during that experiment he was surprised to find a rather large amount of *acetaldehyde*, while as he showed in that paper,[3] he was unable to identify *pyruvic acid*[3] contrary to our expectation.

Then what was that "*pyruvic acid*" in human milk which Arakawa, (one of us) had identified (Dubosq-) colorimetrically?

Indeed Sato[1] (one of us) remarked at the end of his paper on the method with reserve: "The method is, as above described, a colorimetric one, so the the final identification shall be made by strictly chemical method. We shall be able to report such a result in the near future."

The method used by Arakawa seems to be theoretically correct. However there are at least two very important publications on pyruvic acid in human fluids. For one, Toschi et al[5] reported that only about 2/3 of "pyruvic acid" in blood were not due to pyruvic acid itself, the rest being unidentified keto-acids. For the other, Wako[3] was as stated above, unable to identify pyruvic acid in human milk by strict chemical analysis. Though Arakawa seems to have endeavored to exclude other ketoaldehydes, acetaldehyde could not have been excluded completely, especially as their hydrazone will develope red color with KOH, as in case of pyruvic acid. Or some other unidentified ketoaldehydes (Cf. Pyruvate Metabolism)[4] might not have been excluded.

Even then it is interesting that these substances were of low content in human milk positive to Arakawa's reaction and of high content in Arakawa-negative milk.

It is to be emphasized here that *methyl glyoxal*—the most important ketoaldehyde in human milk[3] which will in our opinion cause infantile beriberi,—can not be identified by that method above referred to, owing to the treatment of sodium phosphate and *to exceedingly time-consuming formation and precipitation of its osazone.*

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Clinical Picture of Japanese Encephalitis in Children in 1948

By

Hiroshi Wako, Seizaburo Arai, Naoshi Sasaki, Junichi Ishikawa,
(若生 宏) (新井清三郎) (佐々木直) (石川淳一)
Ototaka Higashi and **Akira Saito**
(東 晉高) (齋藤 章)

(From the Department of Pediatrics, Faculty of Medicine, the Tohoku University, Sendai, Director: Prof. A. Sato and from the Department of Internal Medicine, Prof. Kurokawa's Clinic, Faculty of Medicine, the Tohoku University)

(Received for publication, August 22, 1951)

In 1948, an epidemic of Japanese Encephalitis occurred throughout the country. Sendai with its environs was not excluded. Descriptions of the disease have appeared in a considerable number, especially since 1935. We desire to report our own cases of the disease which prevailed in the summer of 1948 in this region.

Our own cases are 31 in all.

These cases were treated either in the Children's Department of the Tohoku University Hospital or in the Sendai Isolation Hospital.

I. Sex, Age and month of the year

Sex. Of the 31 child patients 23 cases (74.2%) were males and 8 (25.8%) females. Such a predominance of the male sex was already noted in many reports in 1935, and it has been noted too in this year's epidemic by other authors. Of our own cases one male and two female cases were fatal. It is to be noted here that there was not a single case of the disease beside the patient in each family of our own cases.

Age. Of our own 31 cases, the youngest case was a boy of 8 months old. The incidence of age will be seen from Table I (Cf. Table I) which shows 11 cases—the maximum number—between 6 and 9 years of age.

TABLE I

Age Incidence

Age (Years)	0—3	3—6	6—9	9—12	12—16
Number of cases	1	6	11	6	8

This result is concurrent with those of other authors who have described the disease in this year's epidemic.

Seasonal incidence. Of our 31 cases, 21 fell in the month of August, 1 case in July, and the other 9 cases in September. Thus the most cases occurred in August. The very first case was attacked by the disease on July 17 and the very last one on Sept. 24th. The majority were seen in the middle and the last third of the month of August. In Tokyo, the majority are reported to have occurred in the first third of that month. The difference is probably due to that of climate. Most of our own cases were hospitalized on the 3rd, 4th or 5th day of disease. As to contributory causes, there were few cases in which such were suspected. Some cases seem to have being bent on out-door sports, such as base-ball, angling etc.

II. Symptoms

Prodromic symptoms. As the period from infection to onset is very short, it is difficult to make distinctions between prodromic and proper symptoms. According to what the families said, anorexia, fever, headache, malaise etc. were symptoms of some importance if at all; many had a sudden onset with fever.

Symptoms at the onset. Fever occurred in each case. Headache, malaise, convulsions, lethargy were next frequent symptoms.

Temperature. There was no case without fever. 3 types of fever course may be distinguished. One is the type of course in which the onset was a sudden rise of temperature to 39°–40°C, the high fever would continue for about 7 days in order to come down with a crisis. 5 cases were such and mostly clinically slight cases.

The next type is similar to the former type of fever course, the only difference being lytic abatement instead of the crisis occurring in the former type. 15 cases belong to this type with lysis. The third type showed remittent fever—fever of very remittent character—either in the acme or in the stage of fever abatement. The cases belonging to this type were mostly relatively severe. Fatal cases died all during the remittent course of high fever. The duration of high fever was 4 days in 5 cases, 9 days in 1 case, 11 days in 1 case, longer than 12 days in 1 case. It will thus be seen that many cases showed an abatement of fever in about a week which fact was also described in other reports on the epidemics of the disease in 1935 and 1948. The highest temperature recorded was 41.2°C. The cases which showed a fever about 41°C were 3 in number. A majority of the other cases showed a high ever between 39°C and 40°C. 3 cases with the highest temperature were not graver nor were they fatal.

Pulse and Respiration. Pulse was increased corresponding to the fever and there was not a single case of slow pulse. A frequent pulse did not

show any definite relation to the prognosis. Fatal cases showed an irregular and weak pulse prior to death.

Respiration was also increased corresponding to the fever and dyspnea occurred in grave cases. Some of the fatal cases that were accompanied by severe dyspnea showed a relatively good pulse until death.

Facies. Many showed apathetic facial expression, some ailing expression. In febrile stage the face was reddened.

Consciousness. The disorder of consciousness was seen in 24 out of all the 31 cases and occurred during the time from the 4th to the 6th day of disease, thus in the midst of the acme. Severe disorder of consciousness was frequent in grave cases. During the acme stupor and delirium and excitement occurred in the stage of fever abatement, and lethargy prevailed until consciousness was regained in the convalescent stage.

Vomiting. It occurred in 7 cases and in the initial stage it was not frequent. "Caffee dregs like mass" was not vomited. In our own cases vomiting was of less frequence, compared with that in the reports of other authors.

Nuchal rigidity and Kernig's sign: nuchal rigidity occurred in 28 cases, thus in an overwhelming majority of our cases. The grade of rigidity was various. In some cases, it was so severe that it remained still identified in the stage of fever abating and even in the convalescent stage. Kernig's sign was also frequent, though not so frequent as nuchal rigidity. *Knee jerks, ankle clonus and Babinsky's sign:* Knee jerks were lost or weakened in the acme and became exaggerated in the convalescent stage. There were exceptions of course. Ankle clonus and Babinsky's sign were also seen, but infrequent.

Eye symptoms. Conjunctival hyperemia was frequent, but there was no case with a change of the cornea. Pupils were contracted in most cases and the reaction to light was retarded. Ptosis was identified in some cases but there was no case of diplopia.

One case presented peculiar symptoms. It was almost recovered and about to be discharged, when he became irritable and given to crying. The next day the symptoms were aggravated and the patient would not remain lying in bed, but wanted to get up. Then, while these symptoms were getting worse, strabismus, dilatation of the pupils, anisocoria, retardation of pupil reaction to light, tremor of the upper half part of the body, involuntary movements and wryneck occurred too. Ophthalmoscopic investigation (by doctors in the Eye Department of our University Hospital) revealed nothing abnormal in optic disk. He had become taciturn after catching the disease, but suddenly kept muttering all the time and fell off the bed frequently. These symptoms disappeared

some days later. The case was recovered eventually.

Disorders of motors. They occurred in 16 cases. The incidence was about the same as in the epidemic of 1935. Twitching and spasticity of muscles were seen in a fairly large number of cases, and in some of them the spasticity remained to persist even in the convalescent state.

Disorders of speech, trismus, difficulty of deglutition occurred in some cases. But there was no case with flaccid paralysis.

Vesical and rectal disorders. Constipation occurred in 25 cases in the acme of disease and in 20 in the fever abating stage and in 12 in the convalescent stage. Diarrhea was seen in 2 cases. In 5 cases no abnormity of bowel movements was seen. Vesical incontinence occurred in 9 cases in the acme, in 6 cases in the fever abating stage and in 3 cases in the convalescent stage. Rectal incontinence was seen in 4 cases in the acme, in 2 cases in the fever abating stage and in other 2 cases in the convalescent stage. Amounts of serum calcium (Cf. Table II): Serum calcium (Sobel's method) was determined in 7 cases from the 4th day of disease to the 6th, thus during the acme of disease. The average figures were 9.5 mg/dl, thus showing no deviation from those of healthy children. 2 of the 9 cases were fatal, their serum calcium figures being 8.9 and 9.2 mg/dl respectively.

TABLE II

Serum Calcium

Case No.	Age (y=years m=months)	Amounts of Ca (mg/dl)	Day of disease	Prognosis
1	6y 10m	8.9	5	died
2	11y 1m	9.2	4	"
3	7y 8m	8.8 9.0	5 6	Recovered
4	7y 11m	9.7	4	"
5	7y 11m	10.3	4	"
6	3y 3m	9.5	4	"
7	4y 6m	9.6	6	"

Blood glutathione. It was examined in a few cases, but there was no remarkable variation of either O form or R form.

Red sedimentation (Cf. Table III). Yoshida's micromethod was used. The figures were those of one hour. In the acme of disease, it was exceedingly accelerated in 6 cases out of the 16 cases examined, moderately accelerated in 6 other cases. In the fever abating stage it was moderately accerelated in 8 cases out of the 14 cases examined.

TABLE III

Sedimentation Rate of Red Blood Cells

Stage of disease	Acme	Fever abating stage	Convalescent stage
Number of cases / Degrees of acceleration	16	14	29
Most exceedingly accelerated	2	2	0
Exceedingly accelerated	7	1	3
Moderately accelerated	6	8	7
Slightly accelerated	1	3	15
Normal	0	0	4

III. Spinal Fluid

Pressure. Spinal fluid was obtained in the recumbent position of each patient. Many a young child did not cooperate, so that some allowances should be made for the evaluation of obtained figures. The average figures were 240 mm. in the acme stage, 200 mm. in the fever abating stage, and in the convalescent stage they were in the beginning 194 mm. in the middle 174 mm. and in the end of the stage 125 mm., thus gradually coming down toward the normal The result given in Table IV (pressure) is similar to those published by other authors in the epidemics of 1935 and of 1948.

Appearance. The fluid was transparent like water in most cases, but slightly turbid in 7 cases in the acme of disease, in 3 cases in the fever abating stage and in 2 cases in the convalescent stage. Dust-like floating matter and fibrin were seen in some cases, but they were not so striking as seen in the case of tuberculous meningitis.

Globulin (Cf. Table IV). Pandy reaction was positive (in the degree +) in all the cases in the acme of disease. In cases of them it was positive in the degree ##.

In the fever abating stage the reaction was of almost the same intensity. In the fever abating stage the reaction was of almost the same intensity. In the convalescent stage the reaction was still positive, though in a few cases it became weaker to the degree ±.

Nonne reaction was in most cases positive (+) in the acme and in the fever abating stage, but in the convalescent stage it was —, ± or + in each about one third of all the cases.

Cells (Cf. Table IV).

The method of Fuchs-Rosenthal was used.

In the acme in a small majority the count was smaller than 50. In some cases it was over 200. Generally a remarkably large count was not seen, nor did it show a specially increased number in a graver case. As to the proportion

of lymphocytes to polynuclears, a majority of the cases showed a predominace of the former.

TABLE IV
Findings or Spinal Fluids

	Stage of the disease	Acme	Feber abating stage	Convalescent stage
Pressure (mm.)	Number of cases	20	17	26
	Lower than 100	1	1	1
	100—200	7	9	14
	200—300	6	5	10
	300—400	2	1	0
	Higher than 400	2	1	0
Pandy's reaction	Number of cases	14	11	20
	(−)	0	0	1
	(±)	0	1	3
	(+)	12	8	8
	(++)	2	2	8
Nonne's reaction	Number of cases	14	10	20
	(−)	1	2	7
	(±)	1	1	6
	(+)	12	7	7
Cell count	Number of cases	14	12	14
	0—50	5	3	8
	50—100	3	2	6
	100—200	3	2	0
	More than 200	3	0	0

Sugar (Cf. Table V). The average content was 82 mg/dl, perhaps a slight inclination to an increase. It was 87 mg/dl in the acme, 80 in the fever abating stage and 79 mg/dl in the convalescent stage, thus the highest content in the acme. There was probably no relation between the severity of the course and the sugar content.

TABLE V
Sugar Contents of Spinal Fluids

Amount of sugar (mgrm/dl)	Number of cases
Less than 40	1
40—70	2
70—100	4
More than 100	3

Vitamin C. This was estimated in only a few cases; it showed a decrease.

α-naphthol reaction. It was positive in the few cases examined.

Bacteria. Methylene blue and the Ziehl-Gabett method were tried with negative result. Culture was negative too.

IV. Treatment

There is no specific treatment for the disease, though many a method has been recommended. We tried only symptomatic treatment. The method that we considered as of relatively favourable result was hypodermic administration of physiological saline in amounts from 200 to 500 daily combined with intrathecal injection on every other day of thiamine in the amount of 10 mg after removing a certain amount of spinal fluid. This vitamin was also subcutaneously injected daily in amounts of 5 to 20 mgrms. Such a method was reported as efficacious by Nagaki, Yoai and Maeda[9], who had tried the method experimentally and clinically in the epidemic of 1948. Penicillin was ineffective, though we tried it in a few cases.

Remarks. As to the blood picture of these cases, we refer to ' Blood Picture: " Elmonogram " and " Karyogram " of Japanese Encephalitis.'[10]

Conclusions

We observed 31 children cases of Japanese encephalitis who were admitted either in to the Pediatric Department of the Tohoku University Hospital or in to the Sendai Isolation Hospital in the summer of 1948 and obtained the following results.

1) Sex and Age. Of the 31 children patients 23 cases were males and 8 females and thus the male sex was predominant. The highest incidence of age was seen between 6 and 9 years of age (Cf. Table I). The youngest case was a boy of 8 months old.

2) Most of our cases caught the disease in August. The patients were hospitalised mostly on the 3rd, 4th or 5th day of illness. There seemed to be no contributory causes in the history.

3) Many had a sudden onset with fever, headache, malaise, convulsions and lethargy. Three types of fever course may be distinguished, the third type with remittent character in any stage of the disease being clinicilly severe.

The duration of high fever was approximately seven days.

4) Pulse and respiration increased corresponding to the fever, and some of the fatal cases that were accompanied by severe dyspnea showed a relatively good pulse until death. Facial expression was apathetic in many cases. The disorder of consciousness was seen frequently in various degrees corresponding to the severity of the course. Nuchal rigidity and

stiffness of limbs occurred in the majority of the cases. The general convulsion was seen in half a number of the cases. The constipation was seen in many cases.

5) Serum calcium (Sobel's method) showed no deviation in amounts in seven cases examined from those of healthy children.

6) The spinal fluid was, in the acme of the disease, apparently clear and 100–300 mm. in pressure in the recumbent posture and showed an increase of globulin and of leucocytes esp. lymphocytes. The increase of sugar in the stage was not constant.

7) Red sedimentation (Yoshida's micromethod) was accelerated frequently in the acme.

8) The treatment was symptomatic. The hypodermic administration of physiological saline combined with intrathecal injection of thiamin was tried.

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Blood Pictures "ELMoNogram" and "Karyogram" of Japanese Encephalitis in 1948

85th Hematological Paper

By

Hiroshi Wako, Naoshi Sakaki, Junichi Ishikawa and **Ototaka Higashi**
(若生 宏) (佐々木直) (石川淳一) (東 音高)

(From the Pediatric Department, Faculty of Medicine, the Tohoku University, Sendai. Director: Prof. A. Sato)

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The present treatise is the report on the blood picture of 31 child patients of Japanese encephalitis who entered our University Hospital or the Sendai Isolation Hospital in the summer of 1948. The clinical picture is described in another paper of ours.[1] As to judging the quantitative blood picture, Sato and Suzuki's Standard Table of Normal Blood Picture for Every Age[2] was consulted and, to make the white blood picture clear at a glance, the ELMoNogram and the Karyogram devised by Sato and Hayashi[3][4] were used.

Red cells and hemoglobin. Erythrocytes were slightly decreased in number in the acme of disease. The almost same tendence was seen in the periods of abating fever and of convalescence.

Hemoglobin (Sahli's method) showed an increased value in most cases, but in the periods of abating fever and of convalescence a normal value was seen in most cases.

White count. A slight or moderate increase of the count was seen in the acme, except in 3 cases.

ELMoNogram

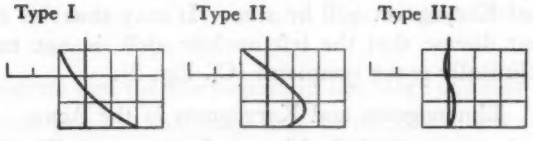

Fig. 1. ELMoNogram of Japanese Encephalitis.

Type I-Elmonogram in the acme. Neutrophils increased to a count 3 or 4 times the normal, whereas lymphocytes showed a decrease and eosinophiles disappeared. There was no remarkable change of monocytes (Fig. 1, Type I).

Type II-Elmonogram in the fever abating stage. The increase of neutrophils became less remarkable, while a striking increase of monocytes was seen. Lymphocytes showed a though slight increasing tendency compared with the acme stage, and eosinophils had begun to reappear (Fig. 1, Type II).

Type III-Elmonogram in the convalescent stage. Neutrophils showed a smaller number within the normal range. A slight increase of monocytes was seen. Lympnocytes which had thus far decreased and eosinophils which had disappeared in the acme have now begun to show a gradual increase (Fig. 1, Type III).

Now Schilling[5] set up, as is well known, a three-phase theory concerning the blood picture in acute infections. Type I corresponds to his first phase "Neutrophilis Combat Phase," Type II to his second phase—"Monocytic Combat Phase" or the intermediate phase and Type III to his third phase—"the post-infectious phase."

As described above, our own cases of Japanese encephalitis have thus shown according to the course three phases corresponding to the three phase theory of blood picture propounded by Schilling in the case of acute infections. Of course there were cases in which the three phases above mentioned did not present themselves quite distinctly.

These cases will be described later (Cf. below).

KARYOGRAM

Fig. 2. Karyogram of Japanese Encephalitis.

In the neutrophilic combat phase of Schilling a remarkable nuclear shift to the left will be seen. In the present disease, however, the left shift not only is not so remarkable, but a strong inclination to early return to the normal Karyogram will be seen. It may thus be characterestic of the present disease that the left nuclear shift is not remarkable in defiance of clinically grave symptoms (Cf. Fig. 2).

Elmonogram and Karyogram in the Acme

Observations were made in 16 out of our cases. The Elmonograms of these may be classed into 3 types-Type I, II and III (Cf. Fig. 3). Of these 16 cases, 12 belonged to Type I, 2 cases to Type II and the remaining 2 to Type III, thus the cases showing Type I being the largest in number. As to the Karyogram, those of the 12 cases belonging to Type I-Elmonogram may be classed into 3 forms (Cf. Fig. 4). To Form I—

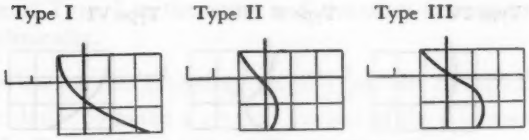

Fig. 3. ELMoNogram of Japanese Encephalitis.

the Form with no inclination of left nuclear shift—belong 5 of the 12 cases belonging to Type I-Elmonograpm, another 5 cases belong to Form II—the Form with a slight inclination of left nuclear shift—and the remaining 2 cases belong to Form III with a rather remarkable left shift. One of these 2 cases died on the 5th day of disease and the other was a grave case that had still remittent fever in the convalescent stage and further showed a severe spasticity of the libms.

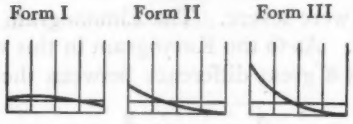

Fig. 4. Karyogram of Japanese Encephalitis.

As to the red sedimentation rate, there was no special relation among the 12 cases belonging to Type I-Elmonogram.

Now for Type II of Elmonogram. Type II ought to be such an Elmonogram as will be seen in the stage of abating fever. The 2 cases belonging to this type showed such an Elmonogram in the acme of disease. They were both of a mild case. It may be imagined that these cases had shown Type I-Elmonogram for a very short time in order to relapse into Type II-Elmonogram very soon, the clinical symptoms being correspondingly mild and not severe.

Type III is similar to Type II, the main difference being a more striking monocytosis. The clinical picture of the cases belonging to the type was not severe one. Or in severity type ranked between the cases of Type I and those of Type II.

The Elmonogram and the Karyogram in the Stage of Fever Abatement

The Elmonogram in this stage may be divided into 3 types: Types IV, V and VI (Cf. Fig. 5), 5 cases belonging to Type IV, another 5 cases to Type V and the remaining 4 cases to Type VI.

Type IV is the type in the Convalescence and of clinically mild course. This will be seen in the total white counts in the cases belonging to this Type (Cf. Type II and Type III). Type V is the Elmonogram quite typical of the stage of fever abatement of acute infectious disease correspond-

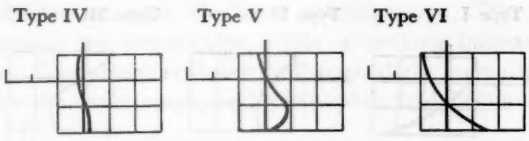

Fig. 5. ELMoNo gram of Japanese Encephalitis

ing to this stage of disease. Clinically the cases belonging to this type were not so mild as those of Type IV, yet took a relatively favourable course.

Type VI, an Elmonogram in the fever abating stage, is similar to Type I in the acme stage, the difference being of occurrence of eosinophils. Cases belonging to Type VI were clinically very severe. Fever was high and continued for along time, showing the remittent type again for a long time in the stage of fever abatement. Disturbance of consciousness and meningitic symptoms were severe. The Elmonogram in these cases seems to change very slowly. As to the Karyogram in this stage, it is like Fig. 6 (A, B). There is not a great difference between these two. B being of

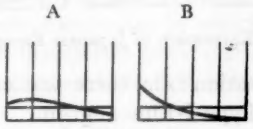

Fig. 6. Karyogram of Japanese Encephalitis.

a slight left shift inclination. No special relation was seen between these two kinds of Karyogram and the Types IV, V and VI of Elmonogram.

The Elmonogram and the Karyogram in the Convalescent Stage

The Elmonograms in this stage were divided into 3 types, Types VII, VIII, and IX (Cf. Fig. 7). Of all the 29 cases, 26 (a great majority)

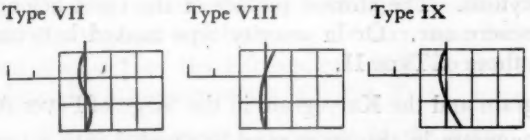

Fig. 7. ELMoNo gram of Japanese Encephalitis.

belonged to Type VII, which is an Elmonogram quite typical of the convelescence of an acute infection almost a normal Elmonogram. 2 other cases belonged to Type VIII, and the remaining case to Type IX. Type VIII is characteristic of eosinophilia and the cases belonging to this type took a clinical course similar to the ones belonging to Type VII. Type IX

remainds one of Type I in the acme and the cases belonging to this type were severe clinically.

Review of the Elmonogram of Japanese Encephalitis

Here we desire to make a general review of the Elmonograms above explained. In Fig. 8, the change of Elmonograms is shown by continuous

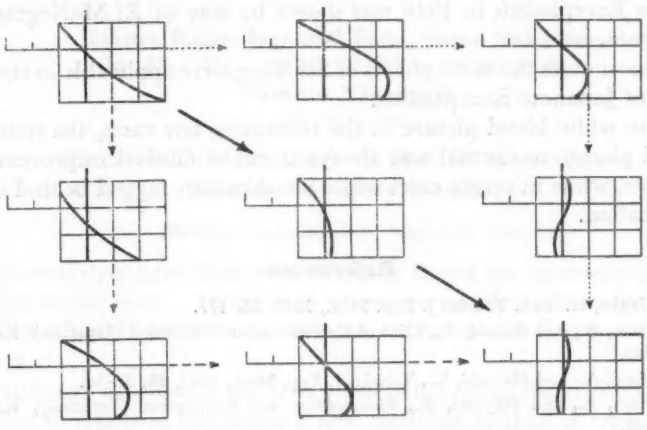

Fig. 8. Review of the ELMo No gram of Japanese Encephalitis.

dotted or broken lines. The changes shown by continuous lines indicate the cases in which the stage of disease and the Elmonogram were typical of an acute infection in general. Most of our own cases belonged to this form.

Now the changes of the Elmonogram shown by dotted lines. In the cases belonging to this form of change, the change of the Elmonogram was always ahead of the stage of disease, so that in the acme of disease and Elmonogram corresponding to the fever-abatement stage and in this stage one corresponding to the convalescent stage would appear. In other words, these cases were mostly clinically mild.

Now, as to the change of the Elmonogram as shown by broken lines. In the cases belonging to this form, the Elmonogram fell behind the stage of disease. In the fever abating stage the Elmonogram corresponded to that in the acme and in the convalescent stage to that in the fever abat-

Fig. 9. Reviews of the Karyogram of Japanese Encephalitis.

ing stage. They were mostly clinically serious cases. The Karyogram showed a change generally corresponding to the stages of disease (Cf. Fig. 9).

Summary and Conclusions

The blood picture of 31 Japanese child patients who suffered from Japanese Encephalitis in 1948 was shown by way of ELMoNogram and Karyogram.

In most cases the three phases of Schilling were applicable to the blood picture of Japanese Encephalitis.

As to white blood picture in the remaining few cases, the restoration of blood picture to normal was always ahead of clinical improvement in mild cases, while in severe cases white blood picture lagged behind clinical manifestation.

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A New Method of Liver Function Test by Double Loading of Azorubin S

By

Isamu Kaito, Sigemiti Tuda, Tatuo Nakazima
(海藤 勇) (津田重通) (中島達雄)
Tamotu Yamaguti
(山口 保)

(From the Medical Clinic of Prof. T. Kurokawa, Tohoku University, Sendai)

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Several dyes have been used clinically to test the excretory functional capacity of the liver. Azorubin S whose use was first advocated by Tada and Nakashima[1] has won as wide an acceptance in Japan as bromsulphalein.

Although azorubin S has been given only once in the previous method, we want to report in this paper a new modified method in which the dye is given in two divided times.

Experimental

Method

2.5 cc. of 1.0% solution of the dye is accurately administered intravenously and the urine is taken hourly during 2 hours, and the same experiment is done without interruption following the last collection of the urine. The percentage reading of excretion for injected amount in each specimen is obtained. In parallel with this test, we performed the previous method after Kanai,[2] urobilinogenuria, Takata's reaction and so on.

In both our and previous methods, the amount excreted is estimated by the Dubosq colorimeter with the standard solution contained an injected amount of dye within 1000, 2000, 3000 cc.—of water.

Results

We summarized the results of experiment performed in 13 normal individuals in Table I.

In the previous method, cases except Case 7 which indicated 1.45% at the third specimen were apparently free from pathologic change.

TABLE I

Normal Cases

No. of case	Sex	Age	Single Method			Double method		
			Output %		Disappearance time (hours)	Output %		Excretory ratio
			3 hours	Total		first 2 hours	last 2 hours	
1	M	20	0.54	10.03	4	12.61	15.86	1.26
2	M	32	0.73	10.42	5	8.97	10.38	1.16
3	F	26	0.68	14.14	5	11.83	14.34	1.21
4	F	17	0.63	10.15	5	11.82	10.85	0.92
5	F	17	0.33	9.26	5	11.23	12.37	1.10
6	M	26	0.84	12.27	5	9.42	11.43	1.21
7	M	35	0.89	12.98	5	10.03	11.78	1.17
8	F	46	1.45	7.81	4	12.57	12.25	0.97
9	M	21	0.22	10.36	4	10.70	10.08	0.94
10	M	36	0.50	10.05	5	13.53	15.64	1.16
11	M	42	0.92	13.30	5	11.80	15.15	1.28
12	F	30	0.13	7.01	4	9.20	8.06	0.88
13	M	25	0.17	10.16	4	9.80	12.63	1.29

On the other hand, in the method introduced by us, the excretion for the first 2 hours was all below 14.0% and excretory ratio (the ratio of the percentage reading for the last 2 hours to that for the first) is below 1.30, ranging from 0.88 to 1.29.

The results of experiment done in 20 cases with liver, gall-bladder and and duct diseases were shown in Table II. 13 out of these cases were indicated as pathological by the previous method. While, we found an increased value for the first 2 hours in 1 case, a rise of ratio in 7 cases and both in 10 cases, showing the liver dysfunction in 18 cases in total by our method. Cases indicating the liver damage by Kanai's method were without exception noticed as pathological by our method.

From the results of experiment done in 20 cases with other various diseases, we found an increased output for the first 2 hours in 6 cases, a rise of ratio in 4 cases and both in one case. After all, 5 out of 6 cases with the disturbance and all out of 6 cases with normal value by Kanai's method indicated the disturbance by our method.

SUMMARY

In azorubin S test, the total excretion in the urine has been widely accepted as the standard of disturbance. However, we consider that its

TABLE II

Cases with Liver, Gall-bladder and Duct Diseases

No. of case	Sex	Age	Diagnosis	Single method			Double method		
				Output %		Disappearance time (hours)	Output %		Excretory ratio
				3 hours	total		first 2 hours	last 2 hours	
1	M	21	Cancer of liver	5.12	30.01	5	14.32	24.40	1.70
2	M	55	"	9.00	54.69	11	23.34	33.70	1.44
3	M	42	"	1.28	14.71	5	12.99	17.55	1.35
4	F	55	"	5.60	55.56	22	21.45	31.27	1.46
5	M	19	Catarrhal jaundice	9.13	57.99	16	37.15	53.32	1.44
6	F	36	"	7.20	52.51	24	21.29	45.92	2.16
7	M	35	"	2.00	33.49	5	15.43	21.47	1.39
8	M	49	"	6.30	48.29	23	19.39	25.96	1.34
9	F	43	Liver tumor	1.42	9.95	5	7.59	10.28	1.35
10	M	45	"	0.87	10.92	4	8.08	10.59	1.31
11	M	42	Clonorchiasis	0.68	11.32	4	10.35	14.14	1.37
12	M	57	Cholelithiasis	0.53	9.98	5	12.24	16.78	1.37
13	M	18	"	0.72	9.95	5	9.11	10.68	1.17
14	F	24	"	1.30	16.65	5	16.24	22.22	1.37
15	F	49	"	6.75	67.99	22	23.85	41.19	1.73
16	M	42	"	5.10	47.99	17	16.39	21.66	1.32
17	M	46	Cholecystopathy	1.56	21.87	5	16.00	17.93	1.12
18	M	45	"	0.55	10.97	5	9.61	13.41	1.40
19	F	52	"	0.56	12.54	5	11.88	15.84	1.33
20	M	58	"	0.63	8.19	4	10.99	11.61	1.06

functional disturbance is possibly estimated in general by the percentage excreted for two hours which was taken as one of the standard in our method.

Moreover, we adopted the excretory ratio as another standard from the point of view that the output for the last 2 hours is slightly more as compared with that for the first in normal and we find a marked increase for the last in cases with liver damage.

It is interesting that we found a rise of ratio in cases in which neither a delayed excretion nor an increased output for the first 2 hours was detected. In these cases it may be considered that a load given for the liver by the first administration causes abnormalities for its function following the second administration. It may be indicative of a latent disturbance of the liver.

Several cases showed normal excretion for the last two hours in spite of an increased output for the first, and it may be possible that the first administration plays a part of stimulus by which its function turned to normal after the second administration.

Further, we can perform the experiment in a shorter time as compared with the classical method in which the urine must be taken generally for a long time in order to observe the total excretion.

Conclusions

1. A new method of the excretory liver function test performed by administration of azorubin S dividing in 2 times is presented.
2. The experiment is done in 4 hours alleviating a load for the patient.
3. We can observe the disturbance of the liver function more sensitively as compared with the previous method.

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Blood Sugar Fluctuation Due to Double Administration of Glucose into the Duodenum

By

Ichiro Uyeda
(植田 一郎)

(From the Medical Clinic of Prof. T. Kurokawa,
Tohoku University, Sendai)

(Received for publication, August 25, 1951)

The two dose test, proposed by Hamman & Hirschman,[1] Staub,[2] and Traugott[3] successively, has found its use for long in testing the carbohydrate assimilating power in patients. Subsequent investigators[4-7] tried to analyse the significance of the double administration of glucose, taken orally, as done by the above named authors, by giving it intravenously. They came generally to see no lesser effect of the second dose.

Another, more close approach to the query, dealt by the latter workers, is, however we believe, to give the test solution into the duodenum directly. The dilution of glucose solution in the stomach and the rapidity of its transportation into the duodenum don't come thus into account.

Now the present writer tried to give a certain amount of the glucose solution once or twice into the duodenun in the subjects, with or without disturbances in the carbohydrate metabolism. The outcome of the two dose test is displayed here in the following paragraphs in short, while that of the one test and the detailed description rê the present report are given elsewhere.[8]

In the early morning after the night fast, 30 g. glucose, solved in 100 cc. distilled water and warmed at 37° C., was introduced into the duodenum by means of the tube. Another 30 g. glucose was given one hour later.

Patients used then to lie down on bed, as quiet as possible, physically and mentally.

A few days after duodenal test, the same amount of the test fluid was given this time orally under the same conditions.

Blood samples, taken from the ear lobule, were assayed of sugar by the method of Fujita and Iwatake.[9]

In healthy person, superfluous to say, the blood sugar concentration increases on the duodenal administration more quickly and extensively than on the oral, just as in the single ingestion test experiment.[8]

Now, between the two kinds of administration, oral and duodenal, there occured invariably an important dissimilarity; the second administration was followed, when given directly into the duodenum, by about the same magnitude of elevation of the blood sugar concentration, while much less marked reaction than the first was always determined in the oral test.

When the glucose solution was orally given in 7 healthy persons, the elevation of the blood sugar content due to the first dose of glucose was 0.03–0.08%, mean 0.064%, and that due to the second—0.003 to 0.06%, mean 0.023%. By way of precaution: The grade of the excess expresses the difference of the maximum sugar concentration brought about by the glucose ingestion over the initial value found just before the administration.

TABLE I

Average Blood Sugar Contents in Healthy and Diabetic Subjects on Giving Glucose Orally or Duodenally

		Before	I Dose			II Dose		
			20′	30′	60′	80′	90′	120′
Healthy (7 persons)	Oral	91	143	156	111	129	123	100
	Duodenal	90	155	151	120	161	143	103
Diabetics (3 persons)	Oral	168	211	248	260	287	302	297
	Duodenal	163	237	268	248	312	341	315

On the duodenal introduction the excess was 0.03–0.1%, mean 0.07% for the first dose, and 0.007–0.08%, mean 0.04% for the second. Or, the excess on the first dose: the second is about 3:1 in the oral test, and 5:3 in the duodenal. And, while in the oral test the hyperglycemic height on the second dose is on an average 0.023% smaller than that on the first, no material difference was measurable between the first response and the second, when duodenally introduced.

With 3 diabetics, the variation was as follows: On the oral administration, the excess was 0.1% (I dose) and 0.05% (II dose) and on the duodenal 0.11% and 0.095% respectively. The ratio of I elevation to II is 2:1 for the oral introduction, and 11:9.5 for the duodenal. The maximum hyperglycemia arrived at by the second dose over that by the first was 0.04% on an average for the oral administration, and 0.07% for the duodenal.

All the figures, thus numerically treated, show that the response to the second administration of the same dose of glucose with the first is always larger in the duodenal test than in the oral, irrespective whether

the carbohydrate tolerance is normal or disturbed. And the difference in the response, expressed by the blood sugar concentration, between the both doses, I and II, was almost the same in healthy and diabetic subjects, i.e. 0.02–0.03%. This fact indicates, therefore, that the smallness of the response to the second ingestion is due largely to the passage of glucose from the stomach into the duodenum.

The data obtained by the oral test, much used clinically, should be thus evaluated with care.

On the other hand, the duodenal test involves all the biological processes from the absorption of glucose untill its appearance in the arterial blood. They are not involved in the case of intravenous test.

Nevertheless the present outcome elucidates a significant figure: In the oral test the smallness of the glycemic effect of the second dose in comparison to the first was found on an average as 0.02–0.03% in the normal individuals, but no difference was observable in the glycemic effect of the glucose ingestion between the first dose and the second, when duodenally introduced. On the other hand, the effect of Staub was invariably negative in the diabetics, irrespective whether the glucose was given orally or duodenally, but the grade of the excessive sugar was different according to the mode of administration, and their difference was computed also as 0.03% mean. The number well coincides with that noted for the normal persons, i.e. 0.02%.

Otherwise expressed, the difference in the grade of the second hyperglycemia due to the different kinds of administration, oral and duodenal, is practically similar in normal subjects and diabetics as well, viz. 0.02–0.03% on an average. The oral administration effects the response to the second glucose ingestion in normal individuals and diabetics in the same magnitude.

Summary

The repeated glucose ingestion test, orally given, has been evaluated by the duodenal test. 30 g. glucose was given to healthy persons and diabetics.

By the oral administration, the hyperglycemia due to the second dose is definitely smaller compared with the first, when tested on the healthy, and the second dose is answered in the diabetics with a greater hyperglycemia than the first, as commonly well known.

The duodenal administration nearly annulled the smaller reaction against the second administration in healthy subjects, known as the Staub effect, and exaggerates the hyperglycemia in the diabetics more strongly than on the oral application.

Be the glycemic effect of repeated glucose ingestion in the normal person and the diabetic properly judged, it makes clear that each kind of application yield almost quite the same outcome in both healthy person and diabetic in relation to the sugar tolerance.

The smallness and retardation of the hyperglycemia due to the oral application, which take place in contrast to the duodenal, appear with a same magnitude in persons with tolerance, normal and lessened as well.

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Influence of Carbohydrate and Fat Diet upon the Carbohydrate Metabolism in Rabbits Poisoned with Alloxan

By

Ichiro Uyeda
(植田 一郎)

(From the Medical Clinic of Prof. T. Kurokawa, Tohoku University, Sendai)

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Introduction

To replace a large amount of carbohydrate by fat and protein in the diet in the treatment of diabetes, will confront with serious problems in the countries as Japan, where carbohydrate food is predominant, not only for patients themselves but for the nation on the whole, particularly at the time-being. Both food and economic conditions are still grave.

From the view-point the diabetic food, proposed by Yamakawa *et al.*[1-4] years ago, which is high in carbohydrate and low in fat, must now more widely circulated than before, we believe. In this country physicians in general are however mostly still unacquinted with the method of Yamakawa, notwithstanding that irrefutable testimonies have been offered by them at that time clinically and experimentally as well.

With this request of the present time in mind, I have compared the influence upon the alimentary glycemia of two kinds of food, one qualified with a richness in carbohydrate and a poorness in fat and another *vice versa*, in rabbits poisoned with alloxan. The drug acts thereupon to bring about a hyperglycemia lasting for several days.

Experimental

Method

Exclusively matured male rabbits were experimented on.

Alloxan was intravenously given in a dose of 200 mg. per kg. body weight as a 5% watery solution. It may be superfluous to say here that the drug effects at first a conspicuous increase in the blood sugar, with disturbances in the liver and kidneys, anemia and injuries in all the body

tissues except the islets of Langerhans. The animal looks seriously. The acute symptoms will then be followed by a long lasting hyperglycemia.

The feeding test was started at the time when the general appearance was well recovered, the good appetite and the height of hyperglycemia showed large fluctuations no more, a matter of two months later.

The routine diet consists for rabbits of 1.8 to 2.5 kg. of 300 g. *tofukara* (bean-curd husk) and 100 g. carrot, with about 240 Cal. and for those of 2.5 to 3 kg. of 350 g., 100 g. and 270 Cal. respectively. The body weight remains then almost unchanged.

The test diet is composed from *tofukara*, carrot, potato and cow's fat. The fuel value being taken as quite the same as the routine diet, the ratio of the food-stuffs was so adjusted as to involve much carbohydrate and less fat and *vice versa*, in the manner shown in the accompanying table. The animal was on the test diet for a certain number of days, say 5–7.

TABLE I

Composition of Various Diets Tested

(For rabbits of 1.8–2.5 kg. The numerals in parentheses: for rabbits of 2.5–3.0 kg.)

1. Normal Diet (per diem)

	Tofukara 300 (350) g.	Carrot 100 g.	Total g.	Total Cal.
Carbohydrate	24 (28)	7.5	31.5 (35.5)	126 (142)
Fat	4.8 (5.6)	0.5	5.3 (6.1)	46 (53)
Protein	15.6 (18.2)	1.5	17.1 (19.7)	69 (80)
			Total:	241 (275)

2. High carbohydrate, low fat diet (per diem)

	Tofukara 200 (250) g.	Carrot 100 g.	Potato 80 g.	Total g.	Total Cal.
Carbohydrate	16 (20)	7.5	15.4	38.9 (42.9)	156 (172)
Fat	3.2 (4)	0.5	0.1	3.8 (4.6)	34 (41)
Protein	10.4 (13)	1.5	0.8	12.7 (15.3)	51 (61)
				Total:	241 (274)

3. Low carbohydrate, high fat diet (per diem)

	Tofukara 150 (200) g.	Carrot 100 g.	Cow's fat 11 g.	Total g.	Total Cal.
Carbohydrate	12 (16)	7.5	—	19.5 (23.5)	78 (94)
Fat	2.4 (3.2)	0.5	11	13.9 (14.7)	125 (132)
Protein	7.8 (10.4)	1.5	—	9.3 (11.9)	37 (48)
				Total:	240 (274)

As the carbohydrate assimilation test, 20% glucose solution was intravenously injected in about one minute in doses such as 0.5 g., 1.0 g. & 1.5 g. per kg. body weight. Before and for three hours after the injection the blood sugar concentration was followed in certain intervals of time. Blood samples from the ear lobe vein was estimated by means of Fujita and Iwatake.[5]

Results

The whole data are epitomized in Table II.

TABLE II

Average of Blood Sugar Content of Rabbits on Glucose i. v. Administration

Doses of Glucose (g. per kg. B.W.)	Diet	Blood sugar (mg. per dl.)					
		Before	5′	30′	1°	2°	3°
0.5 g. (8 cases)	Carbohydrate diet	268	397	—	331	299	274
	Fat diet	264	413	—	344	310	291
1.0 g. (2 cases)	Carbohydrate diet	172	—	366	308	250	196
	Fat diet	170	—	368	325	259	211
1.5 g. (5 cases)	Carbohydrate diet	183	—	403	323	245	175
	Fat diet	160	—	415	346	281	217

The blood sugar content in the alloxan rabbits, determined early in the morning was almost the same, generally speaking, in both groups rabbits, one fed on the high carbohydrate diet, and another on the high fat one. There was detectable however some tendency, though not so significantly, that the individuals fed on the carbohydrate diet have a higher glycemia value than the other, as indicated in the third series of experiments.

Turning now to the intensity of alimentary hyperglycemia, it may be pointed out that there exists a definite discrepancy between both the groups of rabbits, though also not so significant. Namely, the excess due to the intravenous administration of glucose was invariably smaller in the group of carbohydrate diet, through all the doses, while the initial blood sugar level in that group animals was similar to or a little higher than the fat diet group, and never smaller. The table shows it clearly.

Comment

The treatment of diabetics is largely dietetic. It lasts through life. Treatment must therefore be adjusted to the conditions of patients and

environment, and should be so arranged that it can be continued for years without harm, difficulties and inconveniences, physically and economically as well.

The amount of fat in the routine diet of people varies, partly from habit and partly from economic reasons.

And, usually, in those cases where the carbohydrate in the diet is high, the fat is low, and *vice versa*.

A low carbohydrate diet, high in fat, can protect the islands of Langerhans from exhaustion, but, on the other hand, excessive fat can bring about some disturbances in liver tissue, so that the carbohydrate assimilation will be interfered with in the long run, and further the highest fat diet can result in to occasion acidosis in severe diabetics.

On the contrary a moderate carbohydrate diet acts to stimulate the islands mildly, so that they will be protected from the atrophy.

In fact in the countries, overseas, where people are living luxiously and have plenty amounts of every sorts food-stuffs, the specialists, such as Porges & Adlersberg,[6] Brentano,[7] Stolte,[8] Ercklentz,[9] Ellis,[10] etc., have been urging therefore by and by to take a high carbohydrate diet, low in fat. Reference rê the matter under discussion are given, fully in three papers of Yamauti.[11-13]

Summary

Rabbits, poisoned with alloxan, were fed on either a high carbohydrate diet, low in fat or a high fat diet, low in carbohydrate.

Intravenous administration of glucose in certain quantity produced a hyperglycemia there, of a similar strength and duration, irrespective of different kinds of food, or rather the high carbohydrate diet acted to moderate the alimentary hyperglycemia, though only insignificantly.

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Studies on the Antibiotic Substances from Actinomyces

(2nd Report)

On the Classification of Antibiotic Actinomyces by "Streak Plate Method" (So-called "Primary Screening")

By

Masahiko Kuroya, Nobuo Ouchi, and **Masanori Katsuno**
(黒屋 政喜) (大内 信郎) (勝野 正則)

(From the Department of Bacteriology, Faculty of Medicine, Tohoku University, Sendai. Director: Prof. M. Kuroya)

(Received for publication, August 27, 1951)

In the previous paper[1] it was stated, that "streak plate method," which was used at first by Ishida[2] in our laboratory and by Emerson *et al*[3] in other country, was found to be most simple, convenient and sensitive for the isolation and identification of the antibiotic strains of actinomyces. The present paper deals with the results of the experiments, in order to select, identify and classify 360 active strains out of 1800 actinomyces strains using this method, which was called by the authors "Primary Screening."

Experimental

For each strain of actionmyces four plates were employed, two of which were glucose agar plates (A) (meat extract, peptone, glucose, 1% each, Agar 3%) and other two were glucose glycerol agar plates (B) (the former with addition of 1% glycerol). In the centre of these plates the spore suspensions of each strain of actinomyces were inoculated in a form of a circle of 15 mm in diameter and incubated at 27°C for 4 days.

Twelve kinds of test microorganisms (*E. coli* (Umezawa), streptomycin resistant *E. coli* (Umezawa), *Staphylococcus aureus* (F.D.A. 209–P), *B. anthracis* (Umezawa), *Alkaligenes faecalis* on two A plates and *Ps. aeruginosa*, *K. pneumoniae*, *S. sonnei*, *My. avium*, *My. phlei* and *My. smegmatis* on two B plates) were streaked radially around the actinomyces colonies and incubated for 24–48 hours at 37°C.

The lengths of inhibition zones were measured by the nonius in mm and the mean value obtained from each of two plates was considered to represent the inhibitory power of each strain of actinomyces.

Results

By the method mentioned above a "antibacterial spectrum" was shown for each of all strains of actinomyces used in the experiment. Table I shows, for example, those of streptomycin producing strain (*Streptomyces olivaceus*, on which the report will be made afterwards), of streptothricin producing strains (No. 36, No. 24 and Seki), of special substance producing strain (No. 406) and of an actinomyces strain, especially strong inhibitory upon acid-fast bacteria only (No. 364).

TABLE I

Antibacterial Spectra of Several Strains of Actinomyces

Strain of actinomyces	Group	Gram negative bacteria						Gram positive bacteria			Acid fast bacteria		
		Ps. aeruginosa	*Alkalig. faecalis*	*E. coli*	SM resistant *E. coli*	*K. pneumoniae*	*Shigella sonnei*	*St. aureus* 209-P	*B. subtilis* NRRL 588B	*B. anthracis*	*My. smegmatis*	*My. avium*	*My. phlei*
No. 346	I	0	9.0	13.0	0	5.0	13.0	13.5	13.0	17.4	17.5	8.2	11.0
No. 36	I	0	7.7	9.0	9.0	6.7	11.0	11.0	4.5	9.2	10.7	10.9	13.2
No. 24	I	0	10.7	9.8	8.0	6.1	10.5	11.7	10.7	11.0	9.0	11.0	9.5
Seki	I	0	6.7	6.8	6.7	4.0	7.0	8.5	9.3	5.3	5.5	6.0	7.0
No. 406	I	0	11.2	7.7	4.8	0	5.0	7.0	7.5	6.5	6.0	6.8	8.0
No. 17	II	0	0	0	0	0	0	10.7	6.9	7.8	8.7	8.3	8.6
No. 382	III	0	0	0	0	0	0	4.5	3.2	4.8	0	0	0
No. 364	VI	0	0	0	0	0	0	0	0	0	4.0	6.9	6.7

360 strains of active streptomyces were selected by the method mentioned above and classified by their antibacterial spectra into following 4 groups:

I. group: inhibitory upon Gram positive, Gram negative and acid fast organisms ..... 201 (56%) strains

II. group: Inhibitory upon Gram positive and acid fast organisms ..... 130 (36%) strains

III. group: Inhibitory upon Gram positive organisms only..... ..... 14 (4%) strains

IV. group: Inhibitory upon acid-fast organisms only .... 15 (4%) strains

The strains belonging to I group of actinomyces, including streptomycin, streptothricin, chloramphenicoln, aureomycin producingstrains, were the most in number. The strains inhibitory upon Gram negative bacteria

only were not found. The classification mentioned above was not so distinct in the following cases, viz., a strain belonging to II group may appear to belong to III group, if it is low potent, and so forth.

The strains of each group were divided into several subgroups according to their special antibacterial spectra.

I. group. 201 strains belonging to this group were divided as follows:

1) Sta.>coli .....154 strains (including 97 chromogenic and 57 non-chromogenic strains)
   i) Ant>Sub .....76 strains (including 48 chromogenic and 28 non-chromogenic strains)
      a. Coli>O, SM resistant coli=O ...6 strains (all non-chromogenic) (SM producing strains)
      b. Coli≒SM resistant coli>O .....70 strains (including 48 chromogenic and 22 non-chromogenic strains)
   ii) Ant≒Sub, Coli≒SM resistant coli>O .....30 strains (including 13 chromogenic and 17 non-chromogenic strains)
   iii) Ant<Sub, Coli≒SM resistant coli>O .....48 strains (including 36 chromogenic and 12 non-chromogenic strains)
2) Sta≒Coli .....26 strains (all chromogenic)
   i) Ant>Sub.....13 strains
   ii) Ant≒Sub..... 5 strains
   iii) Ant<Sub..... 8 strains
3) Coli>Sta .....19 strains (including 16 chromogenic and 3 non-chromogenic strains)
   i) Ant>Sub..... 6 strains (including 5 chromogenic and 1 non-chromogenic strains)
   ii) Ant≒Sub..... 3 strains (including 2 chromogenic and 1 non-chromogenic strains)
   iii) Ant<Sub.....10 strains (including 9 chromogenic and 1 non-chromogenic strains)
4) Inhibitory upon *Shigella sonnei* only, except Gram positive and acid fast organisms...2 strains (including 1 chromogenic and 1 non-chromogenic strain)

As shown above streptomycin producing 6 strains of actinomyces (Coli>O, SM resistant coli≒O) were easily isolated, one of which was confirmed to belong to *Streptomyces olivaceus*, as will be reported afterwards. Some discrepancies were found between both antibacterial spectra, one pro-

duced by "streak plate method" and the other produced by "cup assay" using culture filtrates or purified antibiotic substances, as will be reported later in other paper. These discrepancies will be probably due to the production of two or more different substances by one and same organism, one of which may scarcely be proved in culture filtrates according to their small amounts of production.

II. Group. 130 strains belonging to this group were divided further into following 3 subgroups:—

1) Ant>Sub.....80 strains (including 36 chromogenic and 44 non-chromogenic strains)
2) Ant≒Sub.....13 strains (including 9 chromogenic and 4 non-chromogenic strains)
3) Ant<Sub.....37 strains (including 21 chromogenic and 16 non-chromogenic strains)

III. group. Active upon Gram positive bacteria only.....14 strains (including 8 chromogenic and 6 non-chromogenic strains)

1) Sta>Ant>Sub.....1 strain (chromogenic)
2) Sta>Sub>Ant.....2 strains (including 1 chromogenic and 1 non-chromogenic strain)
3) Active on Staphyloc. only...4 strains (including 3 chromogenic and 1 non-chromogenic strain)
4) Active on Anthracis only ...5 strains (including 4 chromogenic and 1 non-chromogenic strain)
5) Active on Subtilis only .....2 strains (all non-chromogenic)

IV. group. Active upon acid-fast bacteria only..... 15 strains (including 9 chromogenic and 6 non-chromogenic strains)

The strains of actinomyces belonging to this group inhibited the acid-fast organisms stronger than other bacteria. This characteristic property was proved in their culture filtrates too, showing, for example, the dilution titer of 1:640 against *My. avium*, while no potencies against *Sta. aureus* and *E. coli*.

Kelner and Moton[4] reported previously on "antismegmatis factor" which appeared probably to be a similar substance with those produced by the strains belonging to this group.

Summary

1. The advantages of "streak plate method" for the selection and identification of the antibiotic actionmyces over many other methods lie in its sensitivity, simplicity and characteristic properties, the last of

which enabled us to yield a production of the antibiotic substance or substances by one and same strain of actinomyces in much more number as well as in much more amount in comparison with other methods.

2. Three hundred sixty active strains of actinomyces were classified by their antibacterial spectra into following 4 groups:

I. group: Inhibitory upon Gram positive, Gram negative and acid-fast organisms..... 201 (56%) strains

II. group: Inhibitory upon Gram positive and acid-fast organisms .....130 (36%) strains

III. group: Inhibitory upon Gram positive organisms only..... .....14 (4%) strains

IV. group: Inhibitory upon acid-fast organisms only..... .....15 (4%) strains

The strains of each group were further divided into several subgroups.

3. "Streak plate method," being called by the authors "Primary Screening," is a first step of our systematic investigation of antibiotic substances produced by actinomyces.

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Studies on the Antibiotic Substances from Actinomyces

(3rd Report)

On the Identification by the Specificity of Culture Filtrates of the Antibiotics from Actinomyces

By

Masahiko Kuroya, Nakao Ishida, Shina Kobayashi,
(黒屋 政彦) (石田 名香雄) (小林 しな)
Jiro Konno and **Reiko Chida**
(今野 二郎) (千田 禮子)

(From the Department of Bacteriology, Faculty of Medicine, Tohoku University, Sendai)

(Received for publication, August 27, 1951)

In the first step of our systematic study of antagonistic actinomyces, we have selected 360 active ones out of 1800 strains by the streak plate method using *Sta. aureus* 209–P, *B. subtilis* NRRL 588, *B. anthracis* (Umezawa strain), *E. coli* (Umezawa strain), SM (Streptomycin) resistant *E. coli* (Umezawa strain), *Alkaligenes faecalis*, *Shigella sonnei*, *Ps. aeruginosa*, *K. pneumoniae*, *My. avium*. *My. smegmatis* and *My. phlei* as test bacteria (so-called "Primary screening") and classified them into the following 4 groups.[1)2)]

1st Group	Inhibitory to Gram positive, negative and acid-fast organisms ...	201 (56%)
2nd Group	Inhibitory to Gram positive and acid-fast organisms.....	130 (36%)
3rd Group	Inhibitory to Gram positive bacteria only	14 (4%)
4th Group	Inhibitory to acid-fast bacteria only.....	15 (4%)

As it is scarcely possible and useless to cultivate all strains of active actinomyces on fluid media and to isolate the antibiotics in chemically pure forms, and as it is very reasonable to assume that the nature of the antibiotic substances may be anticipated from the characteristics of culture filtrates of their own parent strains, in other words, to assume the culture filtrates of the active actinomyces as crude water solutions of the final antibiotic substances, and as it seems, therefore, very probable to make an identification of the antibiotics by investigation of specific properties of culture filtrates, we selected 58 representative strains out of 201 belonging to the 1st group actinomyces and cultivated them on the surface of semisolid culture media and their culture filtrates were tested by the follow-

ing several methods of investigations. These procedures will be referred to as so-called "secondary screening" test.

Experimental

1. Culture media and method of cultivation

For the production of antibiotics original Waksman's media (meat extract, peptone and NaCl, 0.5% each, glucose 1% and agar 0.25%) were used and the strains of actinomyces mentioned above were inoculated on the surface of them and incubated at 26–28°C, for 9–12 days, when the pH of culture filtrates reached usually 7.6–7.8 and the antibacterial activity reached almost its maximum.

The selection of the strains showing a high bacteriostatic potency against a special kind of pathogenic microorganisms, and a new device for special culture media to obtain a higher activity of culture filtrate were not undertaken, because the purpose of the present investigation was directed to the identification of the antibiotics contained in culture filtrates and this was able to be conducted by the surface culture using Waksman's culture media, which gave usually a sufficiently good production of antibiotics by almost all strains of actinomyces belonging to the 1st group. If necessary, however, other kinds of culture media, i.e., modified Waksman's media which contained glycerol or starch as carbon sources instead of glucose and shaking culture method were used too.

2. The properties of culture filtrates to be investigated

The culture filtrates, which were obtained at the maximum point of potency and usually at pH 7.6–7.8, were tested on the following four properties.

A) *Comparision of "Terashima" (a strain of Sta. aureus used) dilution unit with E. coli unit*

The culture filtrates were dilutied 2 fold serially by a low nutrient broth, starting from 5–10 fold in case of low potency and from 100–1,000 fold in case of high potency. The total volume of test fluid divided into each tube was 5 cc. One drop (0.05 cc) of 24 hours broth culture of test organisms were inoculated into each tube. A low nutrient broth was composed of 0.5% meat extract and 0.5% peptone, without addition of NaCl, which was suitable to reveal the susceptibility of test organisms very distinctly, because the conditions were rather unfavorable for the growth of test organisms.

In the large majority of cases the strains of the 1st group of active actinomyces were ascertained to belong to the streptomycin-streptothricin-like group.

By preliminary experiments it was revealed that the ratio of inhibitory

potency (dilution unit) against Terashima strain to that against *E. coli* was estimated as 10:1 in the case of streptomycin and as 100:1 in the case of streptothricin-like substance, showing that streptomycin was less inhibitory on *Sta. aureus* (Terashima strain) than streptothricin and streptothricin-like substances. If the culture filtrate of a strain of actinomyces, therefore, showed a ratio of 2:1, for example, in such a test, it was able to assume that the culture filtrate may contain a new antibiotic substance. If necessary, the similar ratio of dilution titer of Terashima strain to that against 209–P strain was calculated too. Streptomycin showed an activity almost similar both to Terashima and 209 strain of Sta. aureus, while streptothricin and allied substances were more active against the former than the latter.

B) Ratio of Terashima dilution unit tested with a medium of pH 7.5 *to that tested with a medium of pH* 6.5

By the similar method mentioned above, dilution units were estimated with a test medium of pH 7.5 and of pH 6.5 respectively and the ratio was calculated. To ascertain the pH, at which an antibiotic was more active, is very essential in order to presume the chemical nature of the antibiotics. As already widely known, penicillin, an organic acid, is more active in acidic pH, than in the alkaline, while streptomycin, an organic base, is more effective in alkaline pH than in the acidic. In the group of steptomycin and streptothricin, the former showed a ratio of 4:1 using a test medium of pH 7.5 and that of pH 6.5 with Terashima strain as test organism, whereas the latter a ratio of 8:1 when estimated by a similar method.

C) Cup assay of culture filtrates, using 12 *kinds of bacteria as test organisms*

Culture filtrates, without being sterilized by heating, were filled in the penicillin cups at the centre of A and B agar plates. A-agar was composed of 1% meat extract, 1% peptone, 0.5% NaCl and 2% agar, while B-agar with further addition of 4% glycerol.

Both A and B agar plates were stored in a refrigerator overnight and 8 kinds of test bacteria, i.e., *Sta. aureus* (209–P), *B. subtilis* (NRRL 588), *B. anthracis* (Umezawa strain), *E. coli*, Steptomycin resistant *E. coli*, *Alkaligenes faecalis*, *S. sonnei* and *K. pneumoniae*, were streaked radially around the cup on the A plate and the remaining 4 kinds of bacteria, *Ps. aeruginosa*, *My. avium*, *My. smegmatis* and *My. phlei*, were inoculated on the B plate in a similar way. After the incubation at 37°C for 24 hours, the lengths of inhibitory zones caused by filtrates were measured in millimeters.

By the method mentioned above antibacterial spectra upon 12 kinds of test bacteria were revealed. Particularly the comparisons of antibacterial activities against *E. coli* and streptomycin resistant *E. coli*, against *B.*

anthracis and *B. subtilis*, and against *Sta. aureus* and *E. coli* enabled us to differentiate streptomycin, streptothricin, and other allied antibiotic substances from each other. The cup assay showed further various diffusibility of antibiotics. By this method it was proved that one antibiotic gave a longer inhibitory zone than the other, in spite of the same dilution titer of both culture filtrates, due to the higher diffusibility of the former than of the latter. This property suggested at least partially the chemical nature of antibiotics too.

D) Anti-Terashima unit of the culture filtrates according to "one-dimen sional diffusion method"

The one-dimensional diffusion method assaying streptomycin was reported by Ishida and others[8] elsewhere. Each culture filtrate was diluted with Sörensen's phosphate buffer (pH 8.0) 2, 4 and 8 fold and superimposed upon the top of the nutrient agar medium which was distributed in small test tubes and inoculated with *Sta. aureus*, Terashima strain and after the incubation at 30°C for 12 hours, the lengths of inhibition zones were measured and the figures were plotted on a semilogarithmic section paper, the lengths of inhibition zones being expressed in millimeters on ordinates and the concentration of culture filtrates in logarithmic number on abscissae.

As already reported, the one-dimensional diffusion method was most sensitive out of several methods of assaying antibiotics. In fact it was often possible to detect the minimum amount of antibiotics in many experiments. Furthermore the grades of the slopes of inhibitory curves obtained by the above-mentioned method showed the diffusibilities of different antibiotics and if two or more inhibitory curves of the same culture filtrate samples on different days were parallel with each other, it may presumably indicate the similarity of the antibiotics produced.

Results

The culture filtrates were in many cases more active in alkaline pH (pH 7.5) and in others vise versa (active in pH 6.5 medium). All strains used in experiments were divided by this test into the following 2 groups:

All strains tested in experiments..... 58 strains

1. Basic group (more active in pH 7.5) ..... 50 strains

This group was confirmed to the main part corresponding to streptomycin-streptothricin group.

2. Non-basic group (more active in pH 6.5) ..... 8 strains

Basic group (Streptomycin-Streptothricin group)

The results of the so-called "secondary screening" test performed on 47 strains belonging to this group are shown in Table I.

TABLE I

Several Properties of the Antibiotic Substances Belonging to Streptomycin and Streptothricin Group

	Terashima / Coli	Terashima / 209-P	pH 7.5 / pH 6.5	Cup assay	Inhibition curve by one dimensional diffusion method* (y=ax+b)	Strains belonging
Streptomycin type	10:1	T≒209-P	4:1	Diffusion (+) Sta≒Coli>0 SM resistent Coli≒0 Sub<Ant.	a=1.0 b=1.5	346, 342, S-39, 304, K-374, K 21-25
Streptothricin type 1	10:1	T≒209-P	4:1	Diffusion (+) Sta≒Coli≒ SM resistant Coli>0 Sub>Ant	a=1.0 b=1.5-2.5	0-36, 39, 0-98, 290, 0-1, 0-20 0-24, 0-93, 0-28, 0-188
Streptothricin type 2	100:1	T>209-P	4:1 to 8:1	Diffusion (0, +) Coli=SM resistant Coli>0 Coli<Sta Sub>Ant	a=0.5-0.8 b=0.5-1.0	0-103, K17, K65, 0-29, 0-89, 90, 0-161, 184, K5, 259, Seki, Itakura, K21-18, K68, K56, 453, 475, 401, 266, 0-188 149, 132, 108, 0-72, 0-26, 0-23, 58, 60, K-27-10

* Inhibition curve by one-dimensional diffusion method: The dilution unit of a filtrate against Terashima strain was estimated and logarithmic numbers corresponding to the unit calculated for 2, 4 and 8 fold dilutions were taken on abscissæ, while the numbers expressing the lengths of inhibition zones in mm. were plotted on ordinates. The inhibition curve expressed the diffusibilities of antibiotics and was obtained in a form of a straight line in a certaine limit of concentration of antibiotics. In the formula y=ax+b a low value of "a" indicated a poor diffusibity, while a low value of "b" showed a relatively low diffusion unit as compared with dilution unit.

As shown in the Table, 47 strains of actinomyces were divided into the following 3 types[4]:

1) Streptomycin type. Six strains were found to belong to this type as shown in the former report.[2] The active culture filtrates were obtained from three strains (346, 342 and S-39) and were investigated by the method mentioned above, being compared with the solution of streptomycin itself.

It was revealed in the case of streptomycin type[2] that the ratio of Terashima dilution unit to *E. coli* dilution unit was about 10: 1, (2) that the ratio of Terashima dilution unit in broth of pH 7.5 to that of pH 6.5 was 4: 1, (3) that by the cup method it was elucidated that the diffusibility of the antibiotics was much higher than the others, (4) that the antibiotics were remarkably active against *E. coli*, while they were completely inactive against the SM-resistant one, (5) that *B. anthracis* was inhibited more profoundly than *B. subtilis*, and furthermore (6) that the inhibitory curve was as similarly steep as SM itself.

A crude hydrochloride of streptomycin was isolated from culture filtrates of Nos. 346 and 342, and further the reineckate of SM (decomposition point: 161–164°C) was obtained from the former.

Besides the strains mentioned above, it is recently found that the strains Nos. K–374 and K 21–25, chromogenic type strains showed some SM-like properties when tested by cup assay. The isolation and purification of the active agent from these culture filtrats are now in progress.

2) Streptothricin type. According to Waksman and others,[5][6] streptothricin is an antibiotic substance which was isolated from the culture filtrate of *Str. lavendulae* and was found to be more inhibitory against a sort of Gram positive bacteria (B. *subtilis*) and almost similarly effective upon *E. coli* in comparison with streptomycin and caused a delayed toxicity and death on injection of 0.5–1.0 mg. into mice weighing 10 g. The procedure of its purification was almost the same as that of SM; its reineckate showed a decomposition at 192–194°C and the empirical formula was found to be $C_{12}H_{25}O_7N_5$.[7]

As there is no preparation that had been confirmed to be streptothricin itself, it is impossible to perform the "secondary screening" tests on streptothricin. After many experiments made upon the culture filtrates of many strains of actinomyces and upon the solutions of purified antibiotics isolated from them, it was taken to be reasonable to divide the streptothricin-like antibiotics into the following two types:

Streptothricin type 1 (ST 1). As indicated in Table I, the streptothricin type 1 resembled very closely the SM in several respects, i.e. in the ratios of Terashima dilution unit to *E. coli* unit, of Terashima unit to 209–P unit, of Terashima unit in broth of pH 7.5 to that of pH 6.5 and in a rapid diffusibility, except that the inhibition zone against *B. subtilis* was longer than that against *B. anthracis* and streptomycin resistant *E. coli* was inhibited in a similar degree as normal *E. coli*.

Out of 10 strains, belonging to the type ST 1, Nos. 0–36, 39, 0–24, 0–93, 0–98 and 0–20 were studied closely on their culture filtrates and especially from the culture filtrate of the strain No. 0–36, an active agent was isolated in crystalline forms of hydrochloride, reineckate and

helianthate, The hydrochloride of No. 0–36 showed no toxic effects, even when injected intravenously into mice in an amount of 20 mg. Inhibitory potency was 1/10 of streptomycin in dilution assay against *Sta. aureus* (Terashima strain) and *E. coli*, 1/4 of the same against *My. tuberculosis* (human type) and as similarly effective as SM against non-pathogenic acid-fast bacilli. This substance was referred to as "Roseomycin" afterwards.

Streptothricin type 2 (ST 2). The active principle of this type was quite contrary to SM in several properties as mentioned above. It was, therefore, provisionally named as "typical streptothricin type," even if it would be somewhat different from the original meaning of Waksman. It was distinctly inhibitory against Terashima strain. The ratio of Terashima dilution unit to *E. coli* unit was 100: 1 and Terashima strain was more strongly inhibited by this type of antibiotics than 209–P strain. It was much more activated in broth of pH 7.5 as compared in broth of pH 6.5. The diffusion was less rapid than the SM and ST type 1, while their antibacterial spectrum in cup assay agreed well with that of the ST type 1. The antibiotic substance of strain No. 259 and Seki was strenuously studied and obtained in a crystalline helianthate.[8)9)] It was highly pontent against almost all kinds of bacteria, although it was somewhat toxic against mice.

Three strains of actinomyces (Nos. 322, 322–2, 322–3 and K-1) showed a characteristic antibacterial spectrum in the streak plate method in the respect, that *E. coli* was more susceptible than *Sta. aureus* (209–*P*). The active agent was more active in alkaline pH and one of which (K-1) was purified in the same way as SM.

Further study indicated, however, that they were producing streptothricin like antibiotics, although they showed somewhat different antibacterial spectra in the streak plate method. This was, therefore, an example of the discomformity between two antibacterial spectra which will be reported elsewhere.

Non-basic group.

The remaining eight strains were found to belong to the non-basic group and divided into the following 3 types:

1) Special type No. 1. Four strains, Nos. 616, 406, 475 and 358, belong to the type, which were very similar with each other in several respects (the production of a wine red, soluble pigment and stronger inhibition against *E. coli* than against *Sta. aureus*). The strain No. 406 was cultivated on the surface of modified Waksman's media containing 1% glycerol instead of glucose and the culture filtrate was shaken with ether in an acidic pH. By this treatment the active substance was removed into ether and if ether layer was shaken with slightly alkaline water, the sub-

stance dissolved again into water. The substance was purified by the repetition of this procedure and the residue after the evapolation of the ether solution was dissolved in acetone and the acetone solution was treated by the chromatographic method. By evapolating the acetone solution that had passed through the Al_2O_3 column, the substance was obtained in a crude crystalline form. Further studies are now being carried on in the laboratory.

2) Special type No. 2. The culture filtrates of two strains (Nos. K-300 and 318) were almost similarly in several respects, even if they were slightly inhibitory against all strains of bacteria, including Gram-negative, positive and acid-fast bacteria. The substances were active against *My. tuberculosis* (human type) too and scarcely adsorbable on charcoal at any pH.

3) Special type No. 3. The culture filtrates of the strains Nos. K 71 and 235 were remarkably highly active in acidic pH and similarly active against Gram-negative and -positive organisms in the cup assay. The studies of these substances are also in progress. Many other types belonging to this group will presumably exist, upon which our studies will be directed.

Summary

1) Fifty-eight representative ones out of 201 strains selected by the streak plate method ("primary screening" test) and proved to be effective upon Gram-positive, Gram-negative and acid-fast bacteria, were cultivated on the surface of Waksman's media and the culture filtrates were tested for their activities and other biological properties by the broth dilution method, by the cup method using 12 kinds of test bacteria, including *E. coli*, streptomycin resistant *E. coli*, *Sta. aureus* (Terashima strain), *Sta. aureus* 209–P and others ("secondary screening" test) and by the one-dimensional diffusion method.

2) By the procedure mentioned above 58 strains of actinomyces were subdivided into several types, which composed of many strains, whose antibiotics were almost identical with each other. The procedure enabled us, therefore, to identify the antibiotics contained in culture filtrates of many strains.

3) Several kinds of known and unknown antibiotics were isolated and purified in crystalline forms. They were found to belong for the most part to the streptomycin-streptothricin group. On the chemical and biological significances of each antibiotic reports will be made by each investigator elsewhere.

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Experimental Studies on the Shwartzman's Phenomenon of the Ear-Labyrinth*

By

Yutaka Tsuiki and **Haruji Kamioka**
(立木　豊)　　(神岡蘇二)

(From the Institute of Oto-, Rhino- and Laryngology, Tohoku University, Sendai. Director: Prof. Y. Tsuiki)

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The pathology of the ear-labyrinth, especially of the non-suppurative labyrinthine disease, has been much discussed in the light of theories of allergy. We have succeeded recently in producing the Shwartzman's[8] phenomenon in the earlabyrinth. This must be of much interest because the cause of the hydrops or hypertonia labyrinthi† can be elucidated from the view point of the biological tissue reactivity to the bacterial filtrate. The details of the experiment are here dealt with.

Method

As the Shwartzman's filtrate B. typhosus culture filtrate was employed as usual. The animals were divided into three groups according to the method of the preparatory injection.

Group 1. comprises 10 animals. 0.5 cc. of the filtrate was injected into the A. vertebralis.

Group 2. comprises 6 animals. The animals were subarachnoidally injected.

Group 3. comprises 2 animals. In these animals the preparatory injection was carried out into the tympanum.

After twenty-four hours a second injection of 1.0 cc. per kilo of body weight of the filtrate was made into the ear vein in all groups of the animals.

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† Since 1947 Tsuiki[10] has performed fenestrating operations on saccus endolymphaticus according to the method described by Portmann on 15 cases. The purpose of the operation was chiefly with view of curing symptoms of vertigo, but other opportunities emerged whereby the method is tried for treatment of tinnitus aurium and hard of hearing. In 12 cases, in whom it was directed against vertigo, good results were obtained with proper follow-up studies upon them having been available. In few cases favorable results were permanently established. In view of these results he is come to believe the existence of a condition what may be known as hypertonia labyrinthi to constituete a syndrome of disease in the labyrinth.

And a few hours or days later the animals were subjected to intravital circulation to obtain microscopic preparations of the labyrinth.

Results

Clinical findings

35% of the animals showed the fall of body temperature, but the remaining animals the rise of body temperature. The Preyer's reflex diminished in 11 cases. Positional nystagmus was observed in all the groups (particularly conspicuous was it in 35% of the animals). Spontaneous nystagmus, in two of the animals which had received subarachnoidal injection. Recession or absence of the compensatory rotation of the eyeballs in 30% of all the animals. Recession or absence of the lift-reaction and other otolith-reflexes in about 45% of all the animals.

To describe typical symptoms in the animals injected through the right A. vertebralis:

In the course of 24 hours following the preparatory injection no change occurred in the state of the animals, slight elevation of body temperature excepted. Postrotatory nystagmus could be induced as before the injection. After the second provocative injection the motion of the animals became dull in 2–3 hours. Duration of the postrotatory nystagmus, short. Positional nystagmus appeared. Lift-reaction etc. absent. Marked fluctuation of body temperature observed.

The animals of the second group (injected subarachnoidally) manifested more conspicuous symptoms. In 3 or 4 hours after the provocative injection rotatory nystagmus was highly increased in some of the animals and it was decreased in others. Positional nystagmus was also observed. In some spontaneous nystagmus happened occasionaly, the eye-balls striking at first to the left and then to the right, extending over 10 minutes. When the animal was forced to walk, it jumped foreward, but soon fel down on the right or left side. Some showed rotation around the longitudinal axis of the body.

Pathological findings

The principal change of the labyrinth consisted in the retention of the lympha and the hyperemia without any inflammation.

According to the method of preparatory injection the grade of change varied. In the second group (injected subarachnoidally) the change was severest. The first group (inject, through A. vertebralis), however, showed also typical findings with retention of the endolympha and hemorrage per diapedesin associated clinically with appearance of positional nystagmus and abnormality of other reflexes.

Extreme changes were seen in the scala media. Especially the stria vascularis exhibited dilatation of capillaries and highly edematous swelling of the epithelium, and the connective tissue of the ligamentum spirale, retention of the lympha. That the dilated capillaries were found to enter the stria vascularis, is very instructive.

Bleeding per diapedesin signifies the sevearest tissue reaction of the area. Extravasation of erythrocytes into the endolymphatic space suggests that the serum simultaneously leaked from the capillaries.

In harmony with the finding above swelling of the Corti's organ and the covering layer and elevation of Reissner's membrane were detected.

In most cases the cells of the Corti's organ uniformly swollen, but in a few cases were found only exclusive swelling of the Corti's membrane and in other few cases degenerative change of the Corti's organ with atrophy of the cells.

Among the epithelial sense cells of the cupula and the macula was retained the lympha. Also the confluence of the fibers of the otolitic membrane was seen.

4 instances showed exudation of cloudy substance in the vestibulum and in the ampulla of semicircular canals, especially in the neighbourhood of the cupula.

The capillaries in the tissues of the cupula and the macula dilated moderately or highly. The grade of dilatation of the capillaries was proportional to the amount of the extravasation, the change of the cells and the clinical manifestations. This findings recall the biological function of the endothels, which after Wittmaack[11] must play a great role in secretion of fluid from vascular system, and the fact, that the dilatation of vessels must have a great influence upon the sense cells, which lie so deep and completely protected from external influences.

Further attention must be paid to the change of ductus and saccus endolymphaticus. The change consisted in distension of the lumen and subepithelial edema of the wall. When strong bleeding took place erythrocytes were seen in the lower part of the saccus or on the epithelium of the wall and in a case 3 days after the second treatment, pigment cells too.

Most of the cases with edema of the scala media and elevation of the Reissner's membrane were associated with distension of the saccus and subepithelial edema, but even when the Reissner's membrane compressed distension of the saccus was recognized. Such findings was usually seen in late cases.

In the peri-lymphatic space hyperemia or bleeding was stronger than in the endo-lymphatic space, especially in the cases after subarachnoidal injection.

The wall of vessels was swellen edematous with extravasation of lymphocytes and eosinophile leucocytes and secretion of fibrin, which is common to the characteristic change of allergy.

Discussion and Conclusion

The autopsy cases of the Meniere's disease were reported by Wittmaack,[11] Hallpike,[8] Rollin,[7] Yamakawa[12] and others. To all of these cases the figure of hydrops or hypertonia labyrinthi can be pointed out as a common finding.

Atkinson,[1] Meyer[6] and others asserted from their clinical experiences the existence of the allergic disease in the auditory organ.

As to the experimental studies on the allergy of the ear Skoog[9] has shown that the carotal syndrom can be elicited in guinea-pigs by means of allergic reaction.

In Japan, Date[2] claimed the allergy of the ear to be a partial symptom of the allergy of the whole body. Besides, Kosokabe[5] studied the influence of low temperature and Kojo[4] that of disorder acid-base equilibrium upon allergy of the auditory organs.

In all of these experimental studies the change of the middle ear was the principal subject.

On the other hand, no account has been as yet made as regards the Shwartzman's phenomenon of the ear-labyrinth.

The present studies have established, that the phenomenon can be produced also in the ear-labyrinth.

The retention of the lympha and other histological findings may be identified with the change in hydrops or hypertonia labyrinthi of man. The present experiment has offered thus some suggestion as to the etiology of the disease called hydrops or hypertonia labyrinthi.

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Fig. 1

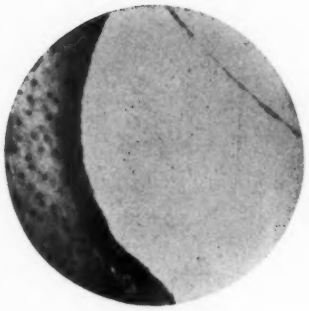

Fig. 2

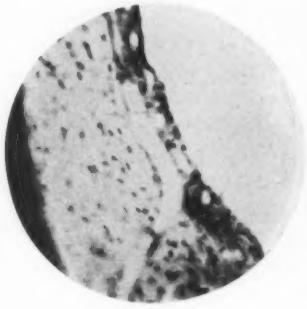

Fig. 3

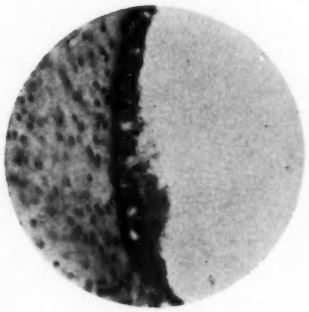

Fig. 4

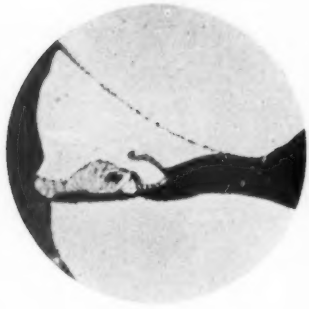

Fig. 5

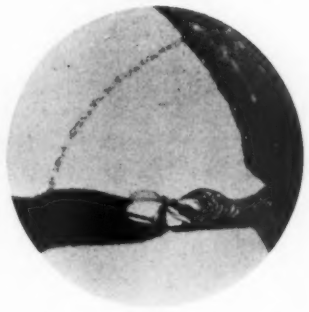

Fig. 6

Fig. 1. Stria vascularis (Control), only the preparatory injection was given.
Fig. 2. Dilatation of capillaries (after the provocative injection).
Fig. 3. The severest reaction (hemorragia per diapedesin).
Fig. 4. Scala media (Control), only the preparatory injection was given.
Fig. 5. Moderate reaction (after the provocative injection).
Fig. 6. The sevearest reaction (conspicuous elevation of the Reissner's membrane).

Fig. 7

Fig. 8

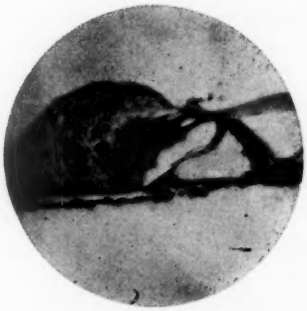

Fig. 9

Fig. 10

Fig. 11

Fig. 12

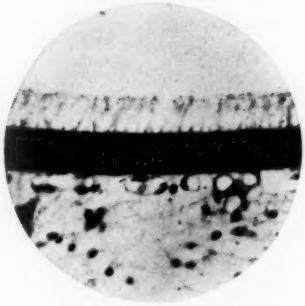

Fig. 7. Swelling of the Corti's membrane.

Fig. 8. Edematous swelling of the Corti's organ.

Fig. 9. Ampulla of the horizontal semicircular canal (Control), only the preparatory injection was given.

Fig. 10 (right ear) and Fig. 11 (left ear). Ampulla after the provocative injection.

Fig. 12. Macula (dilatation of the capillaries in the subepitheliar tissue).

Fig. 13

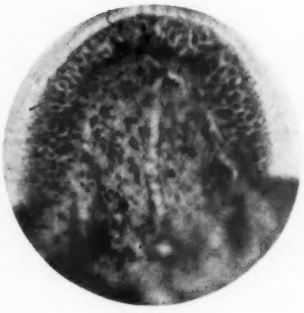

Fig. 14

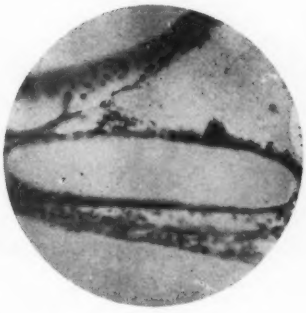

Fig. 15

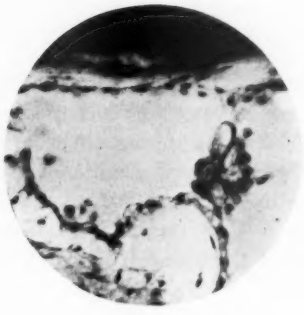

Fig. 16

Fig. 17

Fig. 18

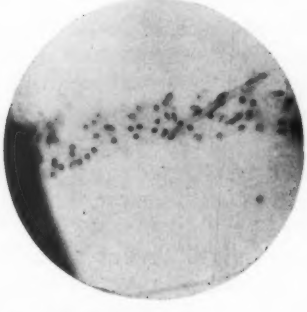

Fig. 13. Crista (dilatation of the capillaries in the subepitheliar tissue).
Fig. 14. Ductus endo-lymphaticus (dilatation of the lumen).
Fig. 15. Saccus endo-lymphaticus (subepithelial edema).
Fig. 16. Dilatation of the saccus-lumen (floating of the erythrocytes and atrophy of the epithelium).
Fig. 17. Erytrocytes on the epithelium of the saccus.
Fig. 18. Dilatation of the capillaries in the Reissner's membrane.

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Influence of Stimulation of the Nasal Mucous Membrane with Ammonia upon the Adrenaline Secretion from the Suprarenal Gland in Non-anesthetized Dogs*

By

Tatsuzi Suzuki, Toshikazu Takagaki, Tsuyoshi Aoki and
(鈴木 達二) (高垣 敏一) (青木 健)
Kahei Tamabuti
(玉澗 嘉平)

(From the Physiological Laboratory of Prof. M. Wada, Tohoku University, Sendai)

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That the cardio-vascular reflex is evoked by the stimulation of the nasal mucous membrane is well known; nevertheless the question whether the adrenaline secretion from the suprarenal gland is concomitantly influenced by the same sort of stimulus has not been satisfactorily decided at the present time. All of the previous investigators dealing with this question used the indirect methods for determining the adrenaline secretion.

Cordier, Magne and Mayer[1] found in rabbits that the pupil, of which the sympathetic innervation was deprived of by removing the upper cervical ganglion and the pupillo-constrictor fibers were paralysed by instilling some drops of 1% atropine solution, responded to the stimulation of the mucous membrane of the upper respiratory tract with a remarkable dilation. This reflex dilation of the pupil was not observed after section of the splanchnic nerves on both sides. From this they inferred that the dilation of the denervated pupil might be due to the augmented adrenaline secretion elicited by the stimulation of the mucous membrane of the upper respiratory tract.

In studying the paradoxical pupil reaction in cats caused by various agencies, Itikawa[2] in our laboratory observed that the dilation of the pupil, denervated by previous removal of the superior cervical ganglion and ciliary ganglion, was elicited by pouring of ammonia solution into the nasal cavity. However in cats, deprived bilaterally of the both suprarenal medulla or of the splanchnic nerves, the dilation of denervated

* Reported at the XXV Annual Meeting of the Japanese Physiological Society at Niigata, 1948, May.

pupil was still causable by the irritation of the nasal mucous membrane with ammonia, though in a small degree.

Using the method of suprareno-jugular anastomosis, Tournade, Chevillot and Chardon[3] ascertained the augmented adrenaline secretion by the application of the same sort of stimulus. The stimulation of the mucous membrane of the nasal and laryngeal cavity of a donor dog with ammonia caused rise in the blood pressure of a recipient dog.

In the present investigation we have used non-anesthetized dogs and studied the influence of the stimulation of the nasal mucous membrane with ammonia upon the secretion of adrenaline from the suparenal medulla with simultaneous recording the arterial blood pressure.

Method

The experiments were carried out on dogs, in which the dorsal spinal roots (D_{11}–L_3) were severed previously on both sides.

By using the method of Satake *et al.*[4] the suprarenal vein blood was sampled through the lumbar way without anesthesia and laparotomy. Adrenaline in the blood samples was estimated by means of the rabbit intestine segment method. The blood pressure was recorded from the femoral artery by a Hg-manometer.

The stimulation of the nasal mucous membrane with ammonia was performed by covering the nose of the dog during a half to one minute with the folded gauze, which was soaked with ammonia solution.

Results

Four experiments were performed with three dogs. The results are presented in the table.

TABLE I

Adrenaline Secretion and Blood Pressure after Application of Ammonia to the Nasal Mucous Membrane

No. of experiment	Time before or after stimulation	No. of specimen	Blood flow per kg. per minute (cc.)	Adrenaline in 1 cc blood (γ)	Adrenaline secretion per kg. per minute (γ)	Blood pressure (mm. Hg)	Heart beat (per min.)	Respiration (per min.)	Body temperature (°C.)
Exp. 1	(23. X. '47. 14.9 kg. D_{11}–L_3 dorsal spinal roots sectioned.)								
27. XI. '47	before								
	13′								38.6
Dog A ♂	9′	1	0.28	0.05	0.01	89	138	27	
15.0 kg.		2	0.27	0.05	0.01				

Application of ammonia to the nasal mucous membrane for 30 seconds

after					88–78–			
0″–30″	3	0.17	0.4	0.07	159–146	120	0–24	
30″–60″	4	0.17	0.1	0.02	146–120	106	15	
60″–90″	5	0.19	0.075	0.02	120–105	116	15	
2′					102	112	18	
4′								38.7

Exp. 2
27. XII. '47
Dog B ♀
8.5 kg.

(5. XII. '47. 9.7 kg. D_{11}–L_3 dorsal spinal roots sectioned.)

before								
17′	1	0.52	0.05	0.03	106	218	18	38.4
	2	0.59						
9′	3	0.50	0.05	0.03	109	222	18	

Application of ammonia to the nasal mucous membrane for 30 seconds

after					119–136–	220– 30		
0″– 30″	4	0.26	2.4	0.62	91–108	40–180	18	
30″– 60″	5	0.78	0.6	0.47	108–138	180	18	
60″– 90″	6	0.38	0.2	0.08	135	180	18	38.1
90″–120″	7	0.61	0.05	0.03	128	210	18	
10′	8	0.50	0.05	0.03	117			
	9	0.42				204	15	38.3
15′					109			

Exp. 3
9. IV. '48
Dog A ♂
12.6 kg.

(23. X. '47. 14.9 kg. D_{11}–L_3 dorsal spinal roots sectioned.)

before								
36′								39.1
33′	1	0.48	0.1	0.05	60	132	30	
	2	0.44						
	3	0.43	0.1	0.04	65	144	30	
12′								39.1

Application of ammonia to the nasal mucous membrane for 60 seconds

after					84–156–			
0″– 30″	4	0.29	0.4	0.12	120	110		
30″– 60″	5	0.35	0.4	0.14	120–107	102		
60″– 90″	6	0.41	0.2	0.08	108	126	18	
90″–120″	7	0.43	0.1	0.04	100	150	16	
120″–150″	8	0.52	0.1	0.05	96	150	12	
10′								39.2
12′	9	0.52	0.1	0.05	88	144	20	
	10	0.44						

Exp. 4
20. X. '48
Dog C ♀
16.1 kg.

(17. IX. '48. 16.5 kg. D_{11}–L_3 dorsal spinal roots sectioned.)

before								
22′	1	0.65	0.05	0.03	71	102	16	38.8
	2	0.60						
9′	3	0.47	0.075	0.04	63	90	12	

Application of ammonia to the nasal mucous membrane for 30 seconds

after								
0″–30″	4	0.29	0.05	0.02	81–175	120–136		
30″–60″	5	0.44	0.05	0.02	175–118	130	24	
60″–90″	6	0.32	0.05	0.02	118–110	132	20	
14′	7	0.48	0.05	0.02	75	102	15	38.8
	8	0.56						

The symptoms developed were almost the same in all dogs. Immediately after the covering of nose with the gauze wetted by ammonia the respiration stopped promptly. The dog twisted his body. Some seconds thereafter vigorous respiratory movements commenced. In some

cases, the urination and repeated regurgitations were observed.

Soon after the start of the stimulation, the blood pressure rose abruptly in Dog A and in Dog C.

In Exp. 1 (Dog A), the blood pressure descended at first from 88 mm. Hg to 78 mm. Hg, then it elevated and 159 mm. Hg was reached about twenty seconds after the start of stimulation and afterwards it fell slowly. In Exp. 3 (Dog A), the blood pressure rose from 84 mm. Hg and reached 156 mm. Hg about twenty seconds after the start of stimulation and afterward it fell gradually. Therefore the magnitude of the blood pressure elevation in Exp. 3 was just the same as in Exp. 1.

Almost the same fluctuation of blood pressure was observed in Exp. 4 (Dog C). The blood pressure rose from 81 mm. Hg after the stimulation and reached 175 mm. Hg about 30 seconds after the start, then it descended gradually. In spite of this remarkable fluctuation of blood pressure, no appreciable change of heart rate was found in Exps. 1, 3 and 4.

Quite different features concerning the heart rate were observed in Exp. 2. Soon after the start of stimulation, a remarkable retardation of heart beat was observed. The initial rate of 220 beats per minute was reduced to 30 beats and it recovered again to 180 beats about 1 minute after the start of stimulation. The blood pressure rose transitorily at first from 119 mm. Hg to 136 mm. Hg and then it fell concomitantly with the retardation of heart beat. 91 mm. Hg was reached about twenty seconds after the start of stimulation, then it rose gradually, reached 130 mm. Hg about one minute after the stimulation and thereafter it descended very slowly. Therefore the change of blood pressure in this case was not so remarkable as in Dog A and Dog C.

As regards the adrenaline secretion rate, its augmentation was observed in Exp. 1 only in the first thirty-second period after the start of stimulation. It increased from 0.01 γ per kg. per minute to 0.07 γ. In the next thirty-second period the adrenaline secretion rate resumed almost its initial rate. Similar result was derived from Exp. 3, which was performed on the same dog as in Exp. 1. In this case, 0.04 γ–0.05 γ per kg. per minute was the initial rate and 0.12 γ, 0.14 γ, and 0.08 γ per kg. per minite were measured in the first, the second and the third thirty-second periods respectively. In the next thirty-second period it returned to the initial rate. In this experiment the stimulation continued for one minute and in Exp. 1 for thirty seconds. The augmentation of adrenaline secretion in Exp. 3 continued somewhat longer than in Exp. 1.

In Exp. 4 the adrenaline secretion rate remained unaltered in spite of the remarkable elevation of blood pressure. In this case 0.03–0.04 γ per kg. per minute was the original secretion rate and after stimulation it was 0.02 γ.

Contrary to the results of Exps. 1, 3 and 4, an enormously augmented adrenaline secretion was observed in Exp. 2. In this case the initial rate of adrenaline secretion was 0.03 γ per kg. per minute. The samples obtained in the first, the second and the third thirty-second periods after application of ammonia showed the augmented adrenaline secretion such as 0.62 γ, 0.47 γ and 0.08 γ per kg. per minute respectively. Thereafter it returned to the initial value.

Summary

Influence of stimulation of the nasal mucous membrane with ammonia upon the rate of adrenaline secretion was studied on non-anesthetized dogs, according to the lumber route method of Satake *et al.* The adrenaline content in the suprarenal vein blood determined by means of the rabbit intestine segment method.

In some cases, in which the blood pressure elevated abruptly after the stimulation of the nasal mucous membrane without any material alteration in the heart rate, the adrenaline secretion augmented slightly or remained unaltered.

In one case, in which the fluctuation of the blood pressure was not so remarkable, but the heart rate decreased markedly after the stimulation, an enormously augmented adrenaline secretion such as 0.6 γ per kg. per minute from one gland was observed.

We should like to express our thanks to Prof. M. Wada for his helpful advice.

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3) Tournade, A., Chevillot, M. and Chardon, G., C. r. Soc. Biol., 1938, **128**, 165.
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Chemistry and Biology of Lipids

IX. Group Lipoid from Hog Lung

(First Paper)

By

Hajime Masamune and **Hidetake Hirata**

(正宗　一)　　(平田 英武)

(From the Medico-chemical Institute, Tohoku University, Sendai)

(Received for publication, August 31, 1951)

We have encountered an unexpected fact that hog lungs involve Group B lipoid notwithstanding that their group glucidamin belongs to Group A as was already mentioned by Utusi.[1] To ascertain if the lungs of any hog possess this characteristic feature we treated the lungs of 20 hogs separately from one animal to another. All the lipid fraction of the materials that had been extracted with hot alcohol and washed with acetone showed Group B activity (anti-iso-hemoagglutination) and the water-soluble part of the tissue residues after the hot alcohol extraction Group A activity (anti-iso-hemoagglutination) except one which was inactive.

The group lipoid was purified by successive fractionations by the aid of organic solvents and water. The final products were electrophoretically homogeneous. The outline of their properties will be described below together with the preparation procedure.

Experimental

Examination of the Group Activity of the Hot-alcohol Extractible Lipid-mixture of Hog Lungs Obtained Separately from One Animal to Another and the Water-extractible Fraction of the Residues after the Hot-alcohol Treatment

Lungs were freed from blood by washing under tap water, and after tearing off of bronchia, masticated. The tissue pulps from each animal were placed together in 4 volumes of denatured alcohol containing 3.5% methanol, squeezed after 5 days and dried in a Faust apparatus.

a) *The lipoid division.* A case, where the dried material from the lungs of an animal amounted to 70 g. altogether, will be taken as an example for the description of further stages. The material was powdered in

an iron motar and extracted with 5 and 3.5 volumes of abs. alcohol in succession by refluxing each time for three hours with following separation, (by decantation and squeeze) of the extract while hot (The tissue residue weighed 62 g. when dried). The transparent dark red fluids were combined and stood for 24 hours, whereby precipitation occurred. The centrifuged supernatant liquid was set aside, and the centrifugate was agitated with 100 cc. of hot abs. alcohol and when cold, again centrifuged. The consecutive supernatant liquids were distilled together in vacuo and the oily dark brown remainder was dried in a desiccator as completely as possible. It was next washed with changes of acetone, until no colored matter came out. On drying, 1.8 g. of a dark brown gelatinous substance were given. Preparations were thus obtained from lungs of 20 hogs. The anti-iso-hemoagglutinative force was determined by our usual procedure.[2] As is illustrated in Table I, all of the preparations inhibited the iso-agglutination of B blood cells at a dilution of 1 : 200.

TABLE I

Anti-iso-hemoagglutinative Potency of the Fraction Extracted with Hot Alcohol and Washed with Acetone of Lungs of 20 Hogs
(The lungs of each hog were treated together)

A and B serum had agglutinin titre 320 and 160 and diluted to 1 : 20 and 1 : 10 respectively.

−: no agglutination, ±: faint aggl., +: slight aggl.,
++: marked aggl., ##: complete aggl.

Specimen No.	Group of erythroc.	Dilution of substances				
		×50	×1 ×10²	×2 ×10²	×4 ×10²	×8 ×10²
1	A	±	+	+	+	++
	B	−	−	−	+	+
2	A	+	+	+	++	++
	B	−	−	−	+	+
3	A	+	+	+	++	##
	B	−	−	−	±	+
4	A	+	+	+	++	++
	B	−	−	−	+	+
5	A	+	+	+	++	++
	B	−	−	−	+	+
6	A	+	+	+	++	++
	B	−	−	−	+	+
7	A	+	+	+	++	++
	B	−	−	−	±	+
8	A	−	+	++	++	++
	B	−	−	−	+	+
9	A	+	+	+	++	++
	B	−	−	−	+	+

10	A	+	+	+	++	++
	B	−	−	−	+	+
11	A	+	+	+	+	++
	B	−	−	−	±	+
12	A	+	+	+	+	++
	B	−	−	−	±	+
13	A	+	+	+	+	++
	B	−	−	−	+	+
14	A	+	+	+	+	++
	B	−	−	−	+	+
15	A	+	+	+	+	++
	B	−	−	−	±	+
16	A	+	+	+++	+++	+++
	B	−	−	−	±	+
17	A	+	++	+++	+++	+++
	B	−	−	−	±	+
18	A	+	++	+++	+++	+++
	B	−	−	−	±	+
19	A	+	+	++	++	++
	B	−	−	−	+	++
20	A	+	++	+++	+++	+++
	B	−	−	−	±	+

b) *The group-glucidamin-containing division.* In the next, the tissue-residuals after the extraction with hot abs. alcohol were subjected to water extraction:— The case, where the material which had been left in the above example was employed, will be described. Of the material (62 g.) 50 g. were shaken with 10 volumes of water (Thymol was added as a preservative) for two days. Centrifuged. The somewhat turbid, orange-colored supernatant fluid was acidified to pH 3.4 with acetic acid (30%) and after centrifuging off of the precipitate, neutralized with NaOH, dialyzed against running water and some deposit thereby was discarded. The solution was distilled in vacuo to about 50 cc., again removed from the occurring deposit and further condensed to about 10 cc., followed by addition of 10 volumes of abs. alcohol. The precipitate here was separated and dried—a white powder, 126 mg. This was taken up in 20 cc. of water and the centrifuged supernatant liquor was concentrated, precipitated and dried similar to above. The white powder given (110 mg.) was washed with ether (Ether was not colored and gave no rest after evaporation). The results of the serological assay on all the products are embodied in Table II. As is seen, 19 products were specifically inhibitory to the isoagglutination of Group A blood cells at a dilution from 1: 400 to 1: 1,600. Since the remaining one (No. 17) was neither Group A- nor Group B-active even at a dilution of 1: 100, it was further washed with hot benzene but again did inhibit neither of the isoagglutinations. The sequels of chemical tests: Molisch +, Goldschmiedt +, biuret +, Osaki

TABLE II

Anti-iso-hemoagglutinative Potency of the Group-glucidamin-containing Fraction of the Hog-lung Residues after Treatment with Hot Alcohol

A and B serum had agglutinin titre 320 and 160 and were diluted to 1:20 and 1:10 respectively.

−: no agglutination, ±: faint aggl., +: slight aggl.,
++: marked aggl., +++: complete aggl.

Specimen No.	Group of erythroc.	Dilution of substances						
		×1 ×10²	×2 ×10²	×4 ×10²	×8 ×10²	×1.6 ×10³	×3.2 ×10³	×6.4 ×10³
1	A	−	−	−	−	±	+	++
	B	+	+	++	++	++	++	++
2	A	−	−	−	−	−	+	+
	B	+	++	++	++	++	++	++
3	A	−	−	−	−	−	+	++
	B	+	+	++	++	++	++	++
4	A	−	−	−	−	±	+	+
	B	+	+	++	++	++	++	++
5	A	−	−	−	±	+	+	++
	B	+	+	++	++	++	++	++
6	A	−	−	−	−	−	+	++
	B	+	++	++	++	++	++	++
7	A	−	−	−	−	±	+	+
	B	+	+	++	++	++	++	++
8	A	−	−	−	−	−	+	++
	B	+	+	++	++	++	++	++
9	A	−	−	−	±	+	++	++
	B	+	+	+	++	++	++	++
10	A	−	−	−	−	−	+	+
	B	+	+	++	++	++	++	++
11	A	−	−	−	−	±	+	++
	B	+	+	++	++	++	++	++
12	A	−	−	−	−	−	+	+
	B	+	++	++	++	++	++	++
13	A	−	−	−	−	−	+	++
	B	+	++	++	++	++	++	++
14	A	−	−	−	−	−	++	++
	B	+	+	++	++	++	++	++
15	A	−	−	−	−	−	+	+
	B	+	+	++	++	++	++	+++
16	A	−	−	−	−	−	+	+
	B	+	+	++	++	++	++	++
17	A	+	+	++	++	++	++	++
	B	+	+	++	++	++	++	++
18	A	−	−	−	−	−	+	+
	B	+	+	++	++	++	++	++
19	A	−	−	−	−	−	+	++
	B	+	++	++	++	++	++	++
20	A	−	−	−	−	−	+	++
	B	+	+	++	++	++	++	++

& Turumi (the direct as well as the indirect) −, phosphorus detected. Three of the products (Nos. 6, 10 and 17) were analyzed. The figures ranged: 7.2–7.5% N, 7.0–9.0% total carbohydrate as glucose (Masamune and Tanabe, First method[8]), 33.8–44.9% ash.

Concentration of the Group B Lipoid

Lung pulps from 10 or 20 hogs were used together at one time. Incipiently the batch was treated as described already. Subsequently the lipid division was fractioned as follows, taking the group activity as an indication. The example below is the case where the balances (30.8 g. in total) after the serological assay of the products from 20 hogs above (31.9 g.) were united to the end in view. 1) The substance was at first divided into the fractions ether-soluble and -insoluble by agitating with many changes of ether (800 cc. altogether) with centrifugation. The ether-insoluble (a white powder) amounted to 8.0 g. 2) The ether extracts united (a dark red, clear fluid) were evaporated under reduced pressure and the residue (22 g., a dark red, hard clump) was treated with cold benzene (800 cc. altogether) as with ether. The benzene-insoluble was a mere trace (40 mg., a white powder). The distilled remainder of the light brown solutions were washed well with acetone and dried, and 21.0 g. of a deep red, mucilaginous product was obtained at this stage. 3) This was taken up in 500 cc. of benzene and after rejecting the small insoluble part, condensed to a thick solution (about 15 cc.) by vacuum distillation and 200 cc. of abs. alcohol added. The precipitate was centrifuged and washed with abs. alcohol (200 cc. in portions). 2.5 g. of a brownish powder were given (**Ppt. I**). The unprecipitated part was recovered by distilling the mother fluid to dryness and after thorough washing with acetone and drying, again taken up in benzene (100 cc.). The benzene-insoluble part (very small) was removed and the solution inspissated to 5 cc. was precipitated with 80 cc. of acetone and washed with changes of acetone. 2.4 g. of a yellow powder were thus yielded—**Ppt. II.** It showed only faint group specificity (Group B-active at a dilution of 1 : 50). 4) Ppt. I was dissolved in 25 cc. of water (pH of the solution 5.2), alkalized to pH 9.6 (0.1 *N* NaOH was used) and shaken with three benzene portions, each equal in volume to the aqueous solution. After the third time the opalescent watery layer was clarified by centrifugation, separated, acidified to pH 1.2 with sulfuric acid (50%), and again shaken with benzene as above. The benzene solutions here were washed well with water. A little NaCl was used in addition when their pH rose to 6.2, because clear-cut separation of the layers after agitation was difficult. Finally the benzene layer was evaporated in vacuo. 5) The remainder (a sticky substance with brown shade) was dissolved in 20 cc. of water (The pH of the solu-

tion was 5.6), acidified to pH 1.2 with sulfuric acid and exhausted with chloroform repeatedly in a just similar manner as with benzene in 4). The chloroform solutions (light brown, clear) were washed with water (NaCl was added when necessary, as above), distilled in vacuo to about 10 cc. and precipitated with 10 volumes of acetone. The deposit was further washed with acetone and dried. 2.1 g. was the brownish powder yielded—**Ppt. III.** This inhibited the isoagglutination of B erythrocytes at a dilution of 1: 2,000.

The intermediate and final products in several runs possessed the group potencies in Table III.

TABLE III

Anti-iso-hemoagglutinative Potency of the Intermediate as well as the Final Products during the Isolation of the Group Lipoid from Hog Lungs

A and B serum had agglutinin titre 320 and 160 and diluted to 1: 20 and 1: 10 respectively.

−: no agglutination, ±: faint aggl., +: slight aggl.,
++: marked aggl., +++: complete aggl.

Intermediate products

Fraction	Group of erythrocytes	Dilution of substances				
		×50	×1 ×10²	×2 ×10²	×4 ×10²	×8 ×10²
Ether-insoluble: Run 1*	A	++	++	++	+++	+++
	B	+	+	+	+	++
Run 2	A	++	+++	+++	+++	+++
	B	+	+	++	+++	+++
Run 3	A	++	+++	+++	+++	+++
	B	+	+	++	++	++
Ether-soluble: Run 1*	A	+	+	+	++	+++
	B	−	−	−	+	+
Run 2	A	+	++	++	+++	+++
	B	−	−	−	+	+
Ppt. I: Run 1*	A	++	++	++	+++	+++
	B	−	−	−	+	+
Run 2	A	+	++	++	+++	+++
	B	−	−	−	+	+
Ppt. II: Run 1*	A	+	+	++	+++	+++
	B	−	±	+	++	++
Run 2	A	++	++	+++	+++	+++
	B	−	+	++	++	+++

Final products

Run No.	Group of erythrocytes	Dilution of substances						
		×1.25 ×10²	×2.5 ×10²	×5 ×10²	×1 ×10³	×2 ×10³	×4 ×10³	×8 ×10³
1*	A	+	+	+	+	++	++	++
	B	−	−	−	−	−	+	+
2	A	+	+	+	++	++	++	++
	B	−	−	−	−	−	+	++
4	A	+	+	+	+	++	++	++
	B	−	−	−	−	−	+	+
5	A	+	+	+	+	++	++	++
	B	−	−	−	−	−	+	+
6	A	+	+	+	++	++	++	++
	B	−	−	−	−	−	++	++

* Run 1 denotes the one whose detailed description has been given in the text.

Properties of the Final Preparations

Electrophoresis: Three of the preparations were examined in a Tiselius apparatus. Only one component was demonstrated after prolonged electrophoresis. Fig. 1 shows the electrophoretic pattern of Prep. No. 1 which was the final product in Run 1.

Fig. 1. Electrophoresis patterns of a 1% solution of the Group B lipoid from hog lung in (a) phosphate buffer, pH 7.8, I=0.29; 20°C; mobility 17.9 $cm^2 \cdot sec^{-1} \cdot volt^{-1} \cdot 10^5$; and in (b) acetate buffer, pH 4.6, I=0.1; 20°C; mobility 16.3 $cm^2 \cdot sec^{-1} \cdot volt^{-1} \cdot 10^5$. Ascending limbs to the left.

Solubilities: The preparations showed same solubilities. They did not dissolve in ether, petrol ether and cold and hot acetone. In methanol and ethanol they did not dissolve in the cold, but dissolved slightly when heated. Completely soluble in chloroform, benzene, tetralin and pyridine. Soluble slightly in cold glacial acetic acid but mostly in hot glacial acetic acid. They dissolved for the greater part in cold water and completely when heated or alkalized (to pH 9.6 with NaOH). From the cold aqueous solution no precipitation took place on acidification (even to pH 1.2) with H_2SO_4.

Qualitative tests: They were Molisch-positive, though faint, but

gave neither biuret nor direct and indirect Osaki & Turumi nor Elson & Morgan reaction.

Chemical analysis: The figures obtained on three of the preparations are recorded in Table IV.

TABLE IV

Composition of Preparations of the Group B Lipoid from Hog Lungs

Preparation (Ppt. III) (Run)	In per cent				In evuivalents per equivalent weight	
	N†	P‡	Total carbohydrate as glucose§	Ash‖	N	P
1*	2.0	4.2	1.3	2.9	1.0	0.94
2	2.0	4.3	1.3	2.2	1.0	0.97
3	2.1	4.3	1.4	1.9	1.0	0.93

* Signify the same as in Table III. † By micro Kjeldahl method. ‡ Haneda, Sinokawa and Yasuoka's modification of Plimmer method. § Masamune and Tanabe (First method). ‖ Pregl's micro analysis without the use of H_2SO_4.

Through the Grant Committee of the Science Council was given a grant from the Education Department in aid to us. H. M.

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1) Utusi, Tohoku J. Exp. Med., 1949, **51**, 101.

2) The readers are referred to Hamasato (Tohoku J. Exp. Med., 1950, **52**, 17) and Oh-Uti (ibid., 1949, **51**, 297) for instance.

3) Masamune and Tanabe, J. Bioch., 1938, **28**, 20.

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Chemistry and Biology of Lipids

X. Group Lipoid of Hog Liver

(First Paper)

By

Hajime Masamune, Tugio Maehara and **Hidetake Hirata**

(正宗一) (前原次夫) (平田英武)

(From the Medico-chemical Institute, Tohoku University, Sendai)

(Received for publication, August 31, 1951)

The procedure of Matuda[1] for concentrating the group lipoids of human liver was applied to hog livers. Diverging from such tissues as the gastric mucosa[2] and lungs[3] from animals of the same species, the livers were found to contain neither Group A lipoid nor Group B lipoid. However, the products (final and intermediate) showed Group O specificity. The most sero-reactive specimen that had been obtained after the whole course of fractionations was investigated regarding its properties, physical and chemical. Electrophoresis at pH 7.8 of that specimen gave a pattern with two boundaries and trials to obtain a pure substance are now being in progress.

Experimental

Fractionation of Hot-alcohol Extractible Lipids of Hog Liver

The procedure whereby Sp. I was prepared (First Run) will be described.

A fresh hog liver weighing 1,687 g. was masticated and after washing with 5 *l.* of physiological saline (on a close linen cloth), covered under 5.4 volumes of 95% denatured alcohol containing 3.5% methanol for 5 days, followed by drying in a Faust apparatus. Of the material dried (370 g.), 270 g. were refluxed with 5 and 3.5 volumes of alcohol in succession, continuing each time for 3 hours. (The tissue residue was set aside for the ether-treatment below.) The hot extracts (dark red, transparent) were allowed to stand for 24 hours in air and filtered. The bulk on the funnel was washed with boiling alcohol and the hot washing was also stood for 24 hours, removed from the precipitate (2.4 g.) and put together with the filtrate above. The fluids combined were distilled, and the residual was dried (a dark brown, extremely viscid fluid, 30 g.) and then exhaustively washed with acetone. 2) The dark brown sticky solid

obtained was treated with four 200 cc. portions of ether. The insoluble part (a white powder) amounted to 4.6 g. 3) The soluble was recovered similar to above and dried and followingly fractioned by repeatedly washing with cold benzene (500 cc. in total). Only 20 mg. of it remained undissolved. 4) The benzene solutions (dark red, limpid) were again distilled and the residue dried (a deep brown clump, 10 g.).

On the other hand, the tissue residue after treatment with boiling alcohol above was subjected to continual ether extraction (with two 400 cc. portions of ether) and the ether extracts (yellowish, clear) were united and evaporated. And the remainder was further subdivided by the aid of acetone (two 40 cc. portions) and benzene similar to above. The distilled residue (a dark brown solid) of the benzene solutions amounted to 0 3 g.

5) The benzene-soluble portions (10.3 g. altogether) were agitated with 5 changes of petrol ether (in total 320 cc.). Almost all the substance went into solution (5 mg. was the insoluble part). 6) The remainder after evaporation of the solution (dark brown, fragile) was washed with portions of 400 cc. acetone. (The yellow washings left 0.3 g. of a light brown semifluid after distillation.) The insoluble part (light brown) amounting to 9.8 g. was divided by means of cold abs. alcohol, employing 800 cc. of the solvent in portions. The alcohol-insoluble part (a dark brown clump, 1.2 g.) was set aside (See 8) below). Again the alcoholic solutions were distilled together in vacuo. 7) On treatment of the residue (a brown fragile substance, 8.5 g.) with hot acetone (450 cc. in total), more than half of it dissolved. The insoluble part here (3.5 g.) was exhausted with cold benzene (300 cc. were used in all) and after rejecting a trace of insoluble substance, the extracts were condensed to a thick solution (about 10 cc.) and precipitated with 300 cc. of acetone—**Ppt. I**, 3.2 g. (This was not further fractioned).

8) The alcohol-insoluble part in 6) was well washed with acetone and thereafter refluxed with acetone repeatedly and when a renewed portion of this solvent remained unstained, the insoluble was dried. This was then precipitated from benzene (All the substance was soluble in benzene) with acetone in the way described in 7) and after washing with acetone (The washing remained colorless), dried—**Ppt. II** (a dark brown powder, 700 mg.). 9) Ppt. II was dissolved in 20 cc. of water, alkalized to pH 9.6 with 0.1 *N* NaOH and shaken with two changes of benzene, each equal in volume to the solution. The watery layer was separated, acidified to pH 1.2 with 50% sulfuric acid (Some precipitate was not separated off) and treated with benzene just as above. The benzene solutions here united (light brown, transparent; pH 2.4) were washed with an equal volume of water three times (The pH of the benzene solution

was raised to 6.2) and distilled under diminished pressure. 10) The product in 9) was now subjected to partition between 20 cc. of water at pH 2.0 and 20 cc. of chloroform and the chloroform solutions (Two chloroform portions were used) were washed with water, condensed to 5 cc. and precipitated with 10 volumes of acetone. The mother fluid had yellow tint. The deposit was washed with acetone thoroughly and dried. 180 mg. of a light brown powder were given—**Ppt. III** (Sp. I).

5 runs of the fractionations were carried out, but the other runs than the one, whose detailed description is given above, were stopped halfway.

Serological Potency of the Fractions

Anti-iso-hemoagglutinative force of the intermediate and final products (1.: the fraction that had precipitated in the cold from the hot-alcoholic extract of tissue, 2.: the ether-insoluble fraction of the hot-alcoholic tissue extracts which had been freed from 1. and washed with acetone, 3.: the part separated from 2. (ether soluble), 4.: the fraction that had dissolved in petrol ether, 5.: the substance after washing 4. with cold acetone, 6.: the substance obtained by distilling the washings of 4., 7.: Ppt. I, 8.: Ppt. II, 9.: Ppt. III) were assayed by our usual procedure. Products 1.–7. were found not to completely inhibit the isoagglutination of either of A and B red cells at dilutions of 1: 25–800 and products 8.–9. at dilutions of 1: 100–1600 (Table I).

Ppts. II's and III's were further examined if they have Group O-specificity. A goat anti-O immune serum[4] (titre 3000) was diluted to 1: 10 and adsorbed by standing with 1/10 volume of Be erythrocytes for 20 minutes, followed by centrifugation. After the adsorption its O-agglutinin titre was reduced to 160 but it did not react with Be erythrocytes at all. The results are shown in Table II.

Physical and Chemical Properties of Ppt. I in First Run

Solubilities

See Table III.

Chemical tests

The product gave the positive Molisch reaction but the negative biuret, Osaki & Turumi (both direct and indirect) and Elson & Morgan reaction.

Composition

It analyzed: N (micro Kjeldahl) 2.84%, P (Haneda, Sinokawa & Yasuoka's modification of Plimmer method) 3.17%, total carbohydrate as glucose (Masamune & Tanabe, First method) 1.24%, ash (micro analysis) 0.6%.

Table I

Anti-iso-hemoagglutinative Force of Fractions of the Hot-alcohol Extractible Lipids from Hog Liver

A and B serum had agglutinin titre 160 and were diluted to 1: 10.

±: faint agglutination, +: slight aggl., ++: marked aggl., +++: complete aggl.

Fraction No.*	Group of erythroc.	Dilution of substances						
		×25	×50	×10^2	×2 ×10^2	×4 ×10^2	×8 ×10^2	×1.6 ×10^3
1 (Run 1)	A	+	++	++	++	+++	+++	
	B	+	++	++	+++	+++	+++	
(Run 2)	A	++	++	++	+++	+++	+++	
	B	++	++	++	+++	+++	+++	
2 (Run 1)	A	+	++	++	++	++		
	B	++	++	++	++	++		
(Run 5)	A	+	++	++	++	++		
	B	+	+	++	++	++		
3 (Run 2)	A	+	++	++	+++	+++	+++	
	B	+	++	++	+++	+++	+++	
(Run 3)	A	+	+	+	++	+++	+++	
	B	+	+	++	++	+++	+++	
(Run 5)	A	+	++	++	+++	+++	+++	
	B	+	+	++	+++	+++	+++	
4 (Run 2)	A	++	++	++	+++	+++	+++	
	B	++	++	++	+++	+++	+++	
5 (Run 2)	A	+	+	++	++	++	++	
	B	+	+	+	++	++	++	
6 (Run 2)	A	+	+	+	++	++	++	
	B	+	+	++	++	+++	+++	
7 (Run 1)	A	+	+	+	+	+	++	
	B	+	+	+	+	+	++	
8 (Run 1)	A			+	++	++	+++	+++
	B			+	++	++	++	+++
(Run 2)	A			+	+	+	++	++
	B			+	+	+	++	++
(Run 3)	A			+	+	+	+	+
	B			+	++	++	++	+++
(Run 4)	A			+	+	++	++	+++
	B			+	+	++	++	+++
9 (Run 1)	A			±	+	+	++	++
	B			+	+	++	++	+++

* Consult the text.

TABLE II

Behaviour of Ppts. II's and III toward the Anti-O Goat Immune Serum Diluted and Treated with Be Erythrocytes

Regarding details see also the text.

−: no agglutination, +: slight aggl., ++: marked aggl.

Ppt.	Dilution of substances						
	×10	×20	×50	$\times 10^2$	$\times 2 \times 10^2$	$\times 4 \times 10^2$	$\times 8 \times 10^2$
II (Run 2)	−	−	−	−	+	+	+
(Run 3)	−	+	+	+	+	++	++
(Run 4)	−	−	−	−	+	+	+
III (Run 1)	−	−	−	−	−	+	+

TABLE III

Solubilities of Ppt. III in First Run

−: insoluble, +: partly sol., ++: mostly sol., +++: completely sol.

Solvent (cold)	Plain water	Water (alkaline)	Methanol	Abs. alcohol	Acetone	Glacial acetic acid	Ether	Chloroform	Benzene	Tetralin	Petrol ether
Grade of dissolution	++	++*	−	−	−	+	−	+++	+++	++	−

* The mixture was alkalized to pH 9.4.

Electrophoresis

Electrophoresis of its 0.7% solution at pH 7.8 showed two boundaries (Fig. 1).

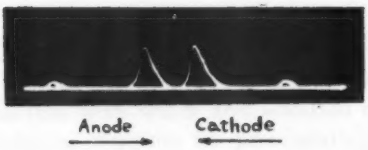

Fig. 1. An electrophoresis pattern of a 0.7% solution of a preparation of the Group O lipoid from hog liver in phosphate buffer, pH 7.8, I=0.29; 20°C; mobility of the faster moving boundary 16.4 $cm^2 \cdot sec^{-1} \cdot volt^{-1} \cdot 10^5$. Ascending limb to the left.

We express gratitude to the Education Department for a grant through the Grant Committee of the Science Council and to Prof. T. Murakami of Forensic Medicine for the anti-O serum. H. M.

References and Notes

1) Matuda, Tohoku J. Exp. Med., 1951, **53**, 333.
2) Readers are referred to Masamune and Siozima, ibid., 1951, **54**, 319.
3) Readers are referred to Masamune and Hirata, ibid., this volume, p. 233.
4) Kindly furnished by Dr. T. Murakami.

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Studies of Tuberculous Pus by Means of Paper Chromatography

First Report

By

Takuo Tamaki

(玉置 拓夫)

(From the Department of Orthopedic Surgery, Faculty of Medicine, Tohoku University, Sendai, Director: Prof. S. Iino)

(Received for publication, September 12, 1951)

To our knowledge there have been reported no precise research about chemical composition of tuberculous pus. I make use of paper-chromatography to the end of determining qualitatively amino acids composition of tuberculous pus plasma protein and free amino acids in its protein free filtrate.

Experimental

Method

1) Preparation of pus plasma protein

Tuberculous pus was obtained from iliac abscess of patients suffering from spondylitis tuberculosa by means of preiliac puncture and rapidly centrifuged. It was then allowed to stand with a large amount of alcohol-ether mixture (1: 1) for a few days and after removal of the supernatant fluid washed with acetone to eliminate fat and phosphatid and the residual dried.

2) Preparation of protein free extracts

An accurate quantity from 5–10 ml of pus plasma were taken and after 5–10 fold dilution with water and addition of a sufficient amount of tungstic acid solution requisite for precipitating all the proteins, the content was vigorously shaken. Then the mixture was allowed to stand at room temperature for 30 minutes. The precipitates were neutralized and evaporated to dryness and further washed with 0.1 ml of 70% alcohol[5] and the centrifugate again dried.

3) Paper chromatographic procedure[1)2)]

The method was followed which had been elaborated in the Bacterio-

logical Institute of Tohoku University for the research of constituents of bacillary body: butanol-acetic acid-water (4: 1: 5) and phenol-water (4: 1) were prepared for agents for development. As a rule, two dimensional ascending method was adopted, using 25×24 cm square of Toyo Filter Paper No. 2. Substantiation was performed by spraying 0.2% ninhydrin butanol solution and subsequent heating at 80°C for 5 minutes.

4) Tungstic acid precipitation[3]

The procedure devised by Folin and Wu for the precipitation of blood plasma protein was modified. To establish the best conditions for the precipitation of pus plasma protein, varied quantities of the precipitating agent of different compositions were examined. Finally 4 ml of the precipitating agent consisting of 2 parts of 10% sodium tungstate and 2 parts of 2/3 N. sulfuric acid were added to 7 fold diluted pus plasma.

5) Hydrolysis of pus plasma protein[1]

1 g each of the materials was hydrolyzed with 10 volumes of 35% sulfuric acid for 48 hours. The hydrolysates, which gave no biuret reaction, were neutralized with $Ba(OH)_2$ and $BaSO_4$ was rejected.

Results and Discussion[12]

Chromatograms of hydrolyzed tuberculous pus plasma protein revealed in 3 cases 13 spots corresponding to aspartic acid, glutamic acid, serine, glycine, alanine, tyrosine, proline, phenylalanine, leucine, valine, lysine, histidine, and arginine (Fig. 1).

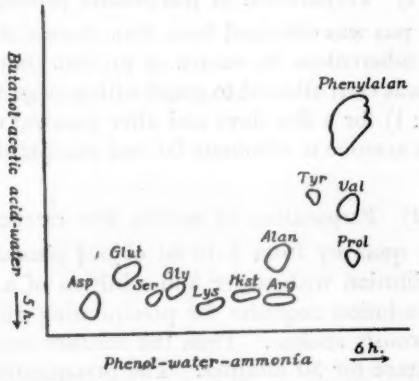


Fig. 1. Butanol-acetic acid-water / phenol-water chromatogram of the complete hydrolysate of the tuberculous pus plasma protein developed with ninhydrin (temp., 20°C).

Asp: Aspartic acid, Glut: glutamic acid, Ser: serine, Gly: glycine, Lys: lysine, Hist: histidine, Arg: arginine, Alan: alanine, Prol: proline, Tyr: tyrosine, Val: valine, Phenylalan: phenylalanine, Leuc: leucine.

While I missed spots of sulfur-containing amino acids such as cysteine, cystine and methionine, it is improbable that this protein substance differs intrinsically from other proteins, in so far as the qualitative amino acids composition concerns.

Chromatograms of tungstic acid filtrates of cold pus plasma from three closed abscesses which had been never before been punctured did not reveal any spot of amino acid, but spots of lower peptides, probably dipeptides (Fig. 2).

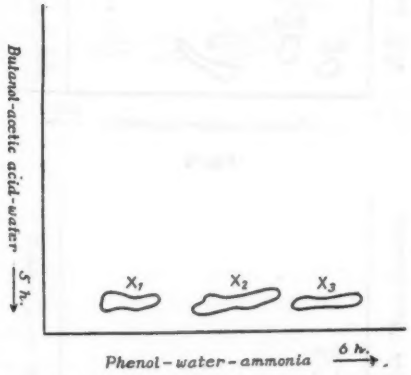


Fig. 2. Butanol-acetic acid-water / phenol-water chromatogram of the tungstic acid filtrate of the tuberculous pus plasma from abscess had been never punctured before developed with ninhydrin (temp., 20°C).
X_1, X_2, X_3: spots of lower peptides.

Maps of tungstic acid filtrates of tuberculous pus plasma from abscesses that had been frequently punctured showed 5 spots corresponding to aspartic acid, glutamic acid, glycine, alanine, and valine with or without spots of lower peptides in 5 cases examined (Fig. 3–7, Table I).

TABLE I

Free Amino Acids in the Protein-free Extracts of Cold Pus Plasma from Abscesses often Punctured Before

No.	Free amino acid
1	Aspartic acid, glutamic acid.
2	Aspartic acid, glutamic acid, alanine.
3	Aspartic acid, glycine, alanine.
4	Alanine, valine.
5	Glycine, valine.

Fig. 3

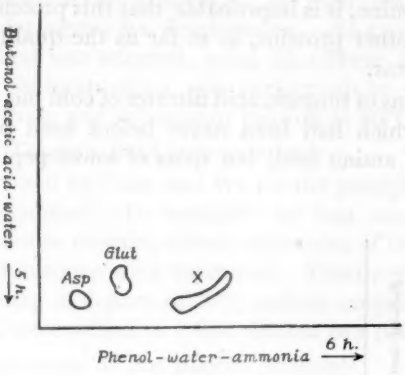


Fig. 4

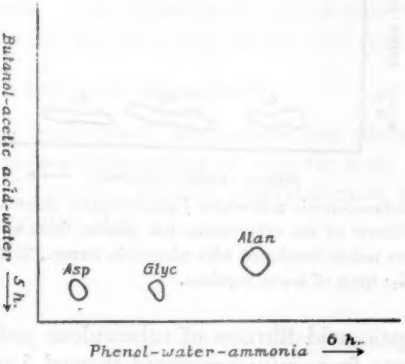


Fig. 5

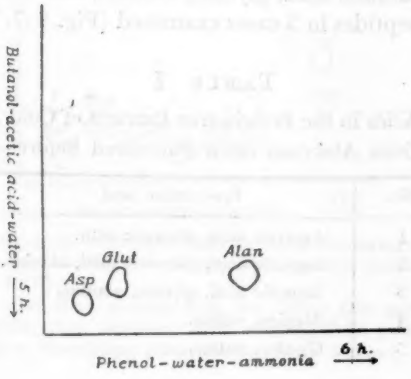


Fig. 6

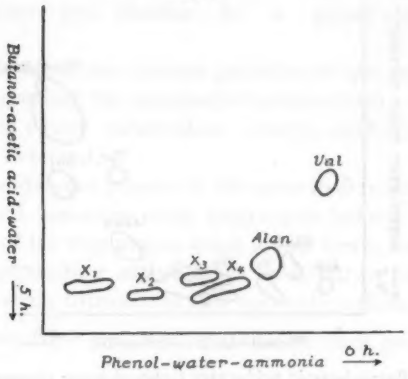
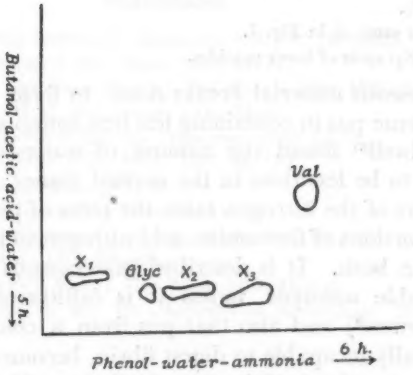


Fig. 7



Figs. 3., 4., 5., 6. and 7. Butanol-acetic acid-water / phenol-water chromatograms of the tunsgtic acid filtrates of the tuberculous pura from abscesses had been punctured before developed with ninhydrin (temp., 20°C).
Notations same as in Fig. 1.
X, X_1, X_2, X_3, X_4: respectively spots of lower peptides.

On the other hand, a chromatogram of tungstic acid extracts of hot pus plasma from acute staphylococcal myositis revealed the presence of aspartic acid, glutamic acid, alanine, tyrosine, valine, leucine, and phenylalanine besides numerous spots of lower peptides (Fig. 8).

There have been expressed many disputes on proteolysis or autolysis of caseous material and tuberculous pus compared with non-tuberculous true pus.

Schmoll[6] analyzed caseous material and found it almost free from proteoses. This indicates that autolysis is slight. According to Müller,[7]

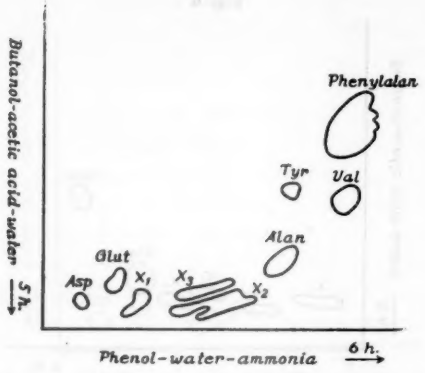


Fig. 8. Butanol-acetic acid-water / phenol-water chromatogram of the tungstic acid filtrate of staphylococcal true pus developed with ninhydrin (temp., 20°V).

Notations same as in Fig. 1.

X_1, X_2, X_3: spots of lower peptides.

even when the caseous material breaks down to form a cold abscess the fluid differs from true pus in containing less free amino acids, e.g., tyrosine is missing. Coldwell[8] found the amount of water-soluble material in caseous tubercles to be less than in the normal tissues, and in the former a much larger part of the nitrogen takes the form of proteose than in the latter. The proportions of free amino acid nitrogen to total nitrogen were about the same in both. It is described that caseous material does not undergo appreciable autolysis unless it is infiltrated with leucocytes (Jobling and Petersen[9]) and also that pus from a cold tuberculous abscess which is usually incapable to digest fibrin, becomes to digest when it is filled with leucocytes by the injection of iodoform (Heile[10]).

I found that closed cold abscess that had not before received a puncture contained no free amino acid in 3 cases, cold abscess frequently punctured, on the contrary, contained free amino acids in 5 cases. The first of these findings offers a chemical evidence that the proteolysis in cold abscess is absent, or if any, very slight, the second suggests probable invasion of leucocytes by frequent punctures. This assumption might not be improbable when collated with Opie's[11] claim that low hydrogen ion concentration in closed cold abscesses is beneficial for leucoprotease activity. Furthermore, I found the true pus had much more free amino acids than the cold pus.

Summary

I investigated amino acids composition of the protein from the tuber-

culous pus plasma in 3 cases and free amino acids in the tungstic acid filtrates of tuberculous pus plasma by a paper-chromatographic procedure.

1) I found tuberculous pus plasma proteins do not essentially differ from usual proteins regarding the qualitative composition.

2) In 3 cases of closed tuberculous abscess never punctured free amino acids were not detected.

3) Free amino acids were present in the protein-free filtrates of tuberculous pura of 5 closed abscesses often punctured before.

4) The number of the free amino acids found there, however, far less than that of the corresponding amino acids in hot true pura. There is a striking contrast between tubercuous and non-tuberculous true pura.

I wish to thank Prof. Iino and Prof. Kuroya for their good advices and cordial guidances.

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Effect of Roentgen Irradiation of the Adrenal Region on the Adrenaline Secretion of the Adrenals in Non-anesthetized Dogs*

By

Tatuzi Suzuki, Toshikazu Takagaki and **Tsuyoshi Aoki**
(鈴木 達二) (高垣 敏一) (青木 健)

(From the Physiological Laboratory of Prof. M. Wada, Tohoku University, Sendai)

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Some experimentalists claimed that the augmentation of adrenaline secretion may be caused by the roentgen irradiation of the adrenal region.

Risse and Poos[1] irradiated the roentgen ray on the upper abdomen of the rabbit, using the pupil sensitized to adrenaline by removing the superior cervical ganglion previously as an indicator of the augmentation of adrenaline secretion. They found after irradiation the dilation of the pupil at first and inferred this dilation to be attributed to the augmentation of the adrenaline secretion from the adrenals.

Using the suprareno-jugular anastomosis method, Zunz and La Barre[2] observed the elevation of the blood pressure and the constriction of the spleen in a recipient dog after the roentgen irradiation in a dose of 100–300 r on the adrenal region of the donor dog. Further, collecting the adrenal vein blood specimens, they measured the adrenaline content in these blood specimens and observed the increase of adrenaline content after the roentgen irradiation of the adrenal area. The same investigators observed also that the pressor effect of the roentgen irradiation of the adrenal region in rabbits was abolished by the removal of the both adrenal glands.

A reduction or an augmentation of the adrenaline content in the adrenal gland caused by the irradiation of roentgen ray was observed by some experimentalists.[3]

It may be added here that some physicians[4] applied the roentgen irradiation of the adrenal region in the treatment of some diseases, assuming that the roentgen ray has an inhibitory effect on the adrenal glands contrary to the reports above quoted.

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The present investigations were undertaken to estimate quantitatively the effect of roentgen irradiation of the adrenal region on the adrenaline secretion in non-anesthetized dogs.

Experimental

Three dogs were used. D_{11}–L_3 dorsal spinal roots were sectioned under morphine-ether anesthesia. About 3 to 6 weeks later the experiments were carried out without being anesthetized or fastened on the table. The suprarenal vein blood was collected by employing the method of Satake *et al.*[5] and the adrenaline content in the suprarenal vein blood specimens was estimated by the rabbit intestine segment method. The blood pressure was registered by the mercury manometer connected with the femoral artery.

The irradiation of roentgen ray on the adrenal region was performed in a dose of 150 r, 200 r and 600 r respectively (160 kv, 2 mA, 1 mm. Al-filter). For the valuable help and advice at the performance of roentgen irradiation, we wish to express our thanks to Dr. S. Miyachi, Assistant Professor of Radiology in our university.

All of the dogs were very quiet during the experiment. No clinical symptoms were observed during and after the irradiation. The blood pressure remained unaltered. The rate of the heart beat and the frequency of the respiration also remained unchanged.

Concerning the adrenaline secretion of the adrenal glands we could not find any definite response to the roentgen irradiation.

In Exp. I, the roentgen ray was irradiated in a dose of 150 r. Before irradiation the rate of adrenaline secretion was 0.009$^-\gamma$ per kg. per min. Just before and just after the end of irradiation it was 0.02$^-\gamma$. Further, from the specimen taken 5 minutes, 15 minutes and 30 minutes after the end of irradiation, the rate of adrenaline secretion was measured as 0.01$^-\gamma$ per kg. per min.

Table I

Effect of Roentgen Irradiation of the Adrenal Region on the Adrenaline Secretion in Non-Anesthetized Dogs

Time	No. of specimen	Blood flow per kg. per min. (cc.)	Adrenaline in 1 cc. blood (γ)	Adrenaline secretion per kg. per min. (γ)	Blood pressure (mm. Hg)	Heart rate (per min.)	Respiration (per min.)
Exp. I. Dog ♀ 8.5 kg. 23. XII. '47. 5. XII. '47 D_{11}–L_3 dorsal spinal roots severed. (9.7 kg.)							
3:15 p.m.	1	0.18	0.05$^-$	0.009$^-$	99	210	18
3:20	2	0.18	0.05$^-$	0.009$^-$	100	210	18

3:25–3:35	Roentgen irradiation on the adrenal region (150 r)						
Just before the end of irradiation	3	0.33	0.05-	0.02-			
Just after the end of irradiation	4	0.33	0.05-	0.02-	94	222	18
3:40	5	0.21	0.05-	0.01-	87	216	18
3:50	6	0.25	0.05-	0.01-	80	216	12
4:05	7	0.21	0.05-	0.01-	84	210	20
4:20					88	210	21
4:35					88	222	18
5:05					92	192	21
5:35					89	216	18

Body temperature 38.9–39.2°C.
Room temperature 12°C.

Exp. II. Dog ♂ 19.7 kg. 25. VI. '48.
12. III. '48. D_{11}–L_3 dorsal spinal roots severed. (16.5 kg.)

4:05 p.m.	1	0.09	0.075	0.007	99	120	36
4:13	2	0.12	0.075	0.009	102	126	36
4:20–5:10	Roentgen irradiation on the adrenal region (600 r)						
5:15	3	0.10	0.075	0.008	104	126	42
5:20	4	0.12	0.1	0.01	98	138	36
5:30	5	0.10	0.1	0.01	95	144	42
5:40	6	0.15	0.1	0.02	98	126	36
5:55	7	0.16	0.1	0.02	96	132	36
6:10	8	0.13	0.1	0.01	99	132	36
6:40	9	0.14	0.1	0.01	96	144	36

Body temperature 39.3–39.9°C.
Room temperature 22°C.

Exp. III. Dog ♀ 16.1 kg. 20. X. '48.
17. IX. '48. D_{11}–L_3 dorsal spinal roots severed. (16.5 kg.)

4:33 p.m.	1	0.34	0.075	0.03	83	108	83
4:40	2	0.45	0.05	0.02	78	114	78
4:50–5:02	Roentgen irradiation on the adrenal region (200 r)						
Just before the end of irradiation	3	0.52	0.075	0.04	86	108	86
5:07	4	0.46	0.075	0.03	80	108	80
	5	0.46					
5:12	6	0.36	0.05	0.02	77	114	77
	7	0.34					
5:22	8	0.46	0.075	0.03	89	114	89
5:32	9	0.47	0.05	0.02	82	102	82
5:47	10	0.25	0.05	0.01	76	132	76
6:02	11	0.27	0.05	0.01	76	132	76

Body tempearture 38.9°C.
Room temperature 18°C.

In Exp. II, the irradiation of the roentgen ray in a dose of 600 r was performed. The initial rate of adrenaline secretion in this dog was 0.007–0.009 γ per kg. per min. The specimen taken 5, 10, 20, 30, 45, 60 and 90 minutes after the irradiation showed that the rate of adrenaline secretion was 0.01–0.02 γ per kg. per min.

In Exp. III, roentgen ray in a dose of 200 r was irradiated. Before irradiation the rate of adrenaline secretion was 0.02–0.03 γ per kg. per

min. Just before the end of irradiation it was 0.04 γ and thereafter it was 0.01–0.03 γ.

Thus we could not find in non-anesthetized dogs any definite effect of roentgen irradiation of the adrenal region upon the blood pressure as well as upon the adrenaline secretion of the adrenal glands.

Summary

The adrenaline secretion rate and the blood pressure were measured in non-anesthetized dogs. Roentgen irradiation of the adrenal region was performed in a dose of 150–600 r. After irradiation the rate of adrenaline secretion and the blood pressure remained unaltered.

Our sincere thanks are due to Prof. M. Wada for giving us helpful suggestions.

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Innervation of Corpus Suprarenale in Human Adult

By

Akira Sato
(佐藤　光)

(From the Anatomical Laboratory of Prof. H. Seto, Faculty of Medicine, Tohoku University, Sendai)

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As the suprarenal gland is, as well known, a very important endocrine organ composed of both embryologically and functionally quite different two parts, the cortex and the medulla, so that histological investigations about the innervation have been done from olden times by many authorities, and that which by Stöhr[15] (1935) is seemed to be most brilliant and trustworthy, though it has some unclarified matters.

So, following Stöhr, in order to give a solution to this problem, I used ten of the suprarenal glands in human adult which were fixed more than a half an year in 10% neutral formol, made them in 40 μ frozen sections and stained them with Seto's silver method used in our laboratory. After having obtained many of very beautiful preparations I have observed them in detail through the microscope and been able to get many of new knowledges as follows.

1. *Innervation of cortex*

Concerning the fine nerve fibres in the cortex of corpus suprarenale we have had since Dogiel many histological works by Riegele,[11] Kolossow,[8] Hirt,[4] Alpert,[1] Pines and Narowtschatowa,[10] Stöhr,[15] Swinyard[16] and etc. Of all of them, however, Stöhr's[15] report is thought to be most trustworthy.

As the nerve elements in the cortex are so extremely fine, that they are able to be found by high power magnification, as Stöhr[15] also asserted strongly, the nerve-staining by the silver impregnation is succeeded never so easily. In addition the nerve fibres in relation to the cortical gland cells are actually quite few. Namely, in spite of the ideal silver staining the nerve fibres passing through between the gland cells are so very few as they can not be compared with those in the medulla, as later described. Though Stöhr[15] did not touch this fact, he would probably accept it enough too. By Swinyard[16] there are found no nerve fibres in the cortex. But his report could not be trusted, because his silver method is not doubtless.

Observing the other endocrine organs conserved in our laboratory, the anterior lobe of hypophysis and the thymus are extremely unfabourable with vegetative nerve fibres, while the medulla of the suprarenal gland, the nonchromaffine paraganglia, the posterior lobe of hypophysis and the epiphysis are very rich in them. It seems, therefore, that the endocrine organs should be divided from the standpoint of nerve supply largely into two groups, the one having only a few of vegetative fibres and the other extremely favoured with them.

In the connective tissue capsule in the surroundings of corpus suprarenale is formed the relative strongly developed nerve plexus, in which are groups of nerve cells of Dogiel's type 1 and type 2 which exist generally in the sympathetic ganglia. Kura[9] described the existence of the arborized and button-formed sensory terminations in the capsel, but I can not but deny it as Stöhr.[15]

As Stöhr[15] and others remarked, such strong nerve bundles (Fig. 1)

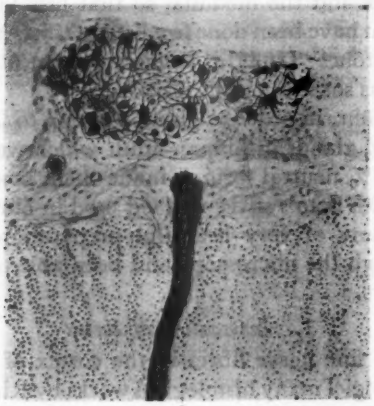


Fig. 1. A sympathetic ganglion in the capsule and a thick nerve bundle passing through the cortex into the medulla. Corpus suprarenale in adult human. Seto's silver impregnation, ×600, reduced to ½.

as could be observed even with the naked eye advance from the plexus capsularis into the parenchyma of cortex. They do not, however, relate to the supply of the cortex, but reach the medulla and pass into the nerve plexus in it. Thus, the cortical cells do not receive the nerve elements from these penetreting nerve bundles. Some of the investigators, however, describe that the nerve elements to supply the cortical cells branch from the plexus capsularis and plexus medullaris. But my observations can not so easily agree with those views, because I can point out that they are represented only by the vegetative fibres running along the small blood vessels or blood capillaries entering and leaving the cortex.

Now, all of the nerve elements entering the cortex along the blood vessels belong to the non-medullated fine vegetative fibres, as shown evidently in the zona fasciculata (Fig. 2) and zona reticularis (Fig. 3), and their

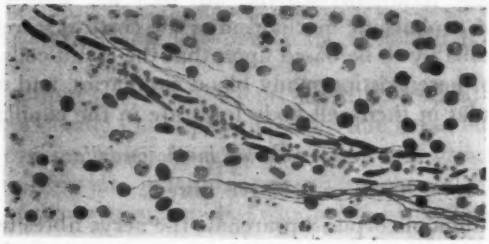


Fig. 2. Finest vegetative nerve elements in the zona fasciculata of the cortex. Corpus suprarenale in adult human. Same staining, ×600, reudced to $\frac{1}{2}$.

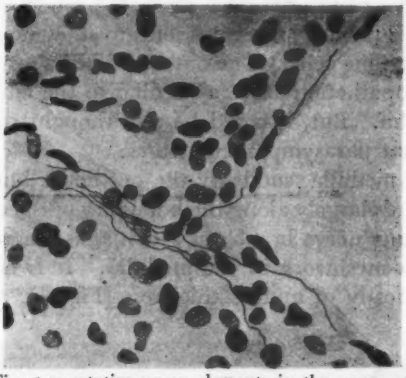


Fig. 3. Finest vegetative nerve elements in the zona reticularis of the cortex. Corpus suprarenale in adult human. Same staining, ×1000, reduced to $\frac{1}{2}$.

ending is represented by the terminalreticulum which has been proved by many investigators since Stöhr (1932) in various places in the body. This terminalreticulum, however, differs a little in structure from such a typical, extremely fine neurofibrillar reticular cord as found in common blood vessels or in smooth muscle tissue. It shows, namely, in general few branching and anastomosing, and Schwann's leading-plasmodium also is not so evident, that Schwann's nuclei are rarely seen. Such a terminalreticulum supplies the gland cells in a contact mode, without advancing into their cell body. These views are similar to those of Riegele[11] and Alpert[1] and agree with those of Stöhr[15] mostly. Besides, this terminalreticulum also never terminates freely, so that such free endings showing button-like

bodies as Pines and Narowtschatowa[10] described must be denied with Kolossow[8] and Stöhr.[15]

It would be noticed finally, that the cortical cells do not always receive the supply of the terminalreticulum, but they are mostly free from it. This fact would explain the mechanism of the cortical secretion as follows. It is seemed probably to be based upon that the gland cells are pressed by the extension of here existing many blood capillaries, and accordingly the secretory granules in their cell body infiltrate in the capillaries.

2. *Nerve elements in the medulla*

a. Nerve fibres

In the medulla of corpus suprarenale the nerve fibres take their rise in the nerve bundles coming from plexus capsularis and passing through the cortex into the medulla, as above mentioned, as well as in the sympathetic nerve cells existing in the medulla, as later described. Besides, there is the vegetative nerve plexus running along the blood vessels entering the medulla, too. This plexus, however, seems to show no relationship to the medullar or chromaffine cells. The out-nerve fibres for the medulla largely might be of sympathetic nature according to the embryological nature recognized by Kohn. But, it could be also thought that there might be contained somewhat parasympathetic fibres.

Reaching the medulla, suddenly the out-nerve bundles change their course, run forewards largely along the boundary of the cortex and medulla and divide into many nerve branches, which anastomose in various modes and form finally a meshwork in the medulla. It is noticeable, that the nerve fibres are mostly of middle thickness (Fig. 4), as Stöhr[15] also re-

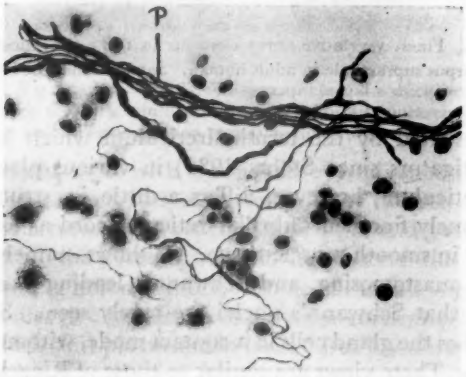


Fig. 4. Nerve elements in the medulla of corpus suprareale in adult human. p a small nerve bundle forming plexus medullaris. Same staining, ×600, reduced to ½.

marked, in spite of the rule that the sympathetic fibres become generally finer with the approach of the terminal parts.

From this plexus medullaris, furthermore, appear numerous and finer nerve branches which show each special winding, subdividing and anastomosing, and distribute more or less equally in the parenchyma. It is easily recognized even with low power magnification how many nerve fibres exist here.

By the way, observing the distribution of the terminal nerve fibres for the gland cells in detail, after separating from fine nerve bundles they take each a lonely course, showing a special winding and variety of thickness, and sometimes divide into much thinner subbranches often showing likely the increasing of thickness and special winding in places (Figg. 4, 5 and 6).

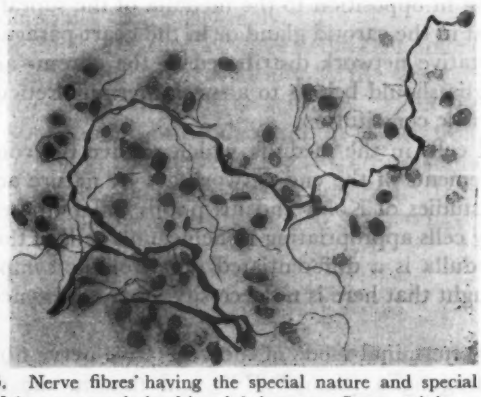


Fig. 5. Nerve fibres' having the special nature and special winding. Medulla of the suprarenal gland in adult human. Same staining, ×600, reduced to ½.

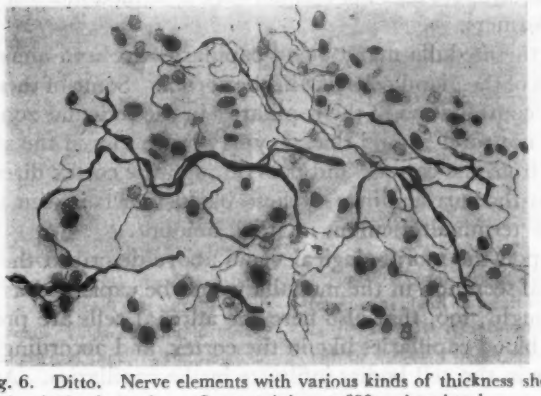


Fig. 6. Ditto. Nerve elements with various kinds of thickness showing a typical reticular formation. Same staining, ×600, reduced to ½.

It would be asserted here strongly, that neither such plate- and button-like free terminations as described by Pines and Narowtschatowa,[10] Dogiel,[2] Kolossow[8] and etc. nor such fine reticular bodies terminating in the gland cells as de Castro, Riegele and Boeke reported are the terminations of the terminal fibres in the medulla. After all, every terminal nerve fibre does never terminate freely, but always anastomoses each other, to make as a whole a large reticular system.

Stöhr[15] described that this reticular endsystem should also belong to the terminalreticulum distributed in other common tissues. I also support it to call the terminalreticulum. But, as in terminal mode it differs from the common terminalreticulum composing of finest neurofibrillar network remarkably and resembles to that in the non-chromaffine paraganglia standing in opposition to the medulla of the suprarenal gland, for example, to that in the carotid gland or in the heart-paraganglia, extremely, so the vegetative network distributed in the chrom- and non-chromaffine paraganglia should belong to a special terminalreticulum according to its characteristic constitution.

The gland cells in the medulla which receive the contacting supply of the nerve elements of the end-network are by nature according to the embryological studies of Kohn and his pupils differentiated from the so-called accessory cells appropriating to Schwann's cells of the sympathetics, so that the medulla is a differentiated organ of the sympathetic nerves. Then, it is thought that here is no necessity for the existence of Schwann's cells.

Between the terminal mode of the vegetative nerve fibres in the non-chromaffine paraganglia (Seto)[16b][17][18] and that in the medulla of the suprarenal gland is not so large difference, but in the latter the nerve fibres are somewhat stronger and the end-network is formed more or less rougher than in the formers.

Also in the medulla many blood capillaries are seen among the cords of the groups of the gland cells like in the cortex. Some of them are shown as the direct continuation of the capillaries in the reticular zone of the cortex, but it is not rarely found that they have their rise in the small arteries which come from the capsule and pass through the cortex directly into the medulla. In the surroundings of these blood vessels is seen the common neurofibrillar terminalreticulum in small amount.

Based upon the morphological views above described the mechanism of the adrenal secretion in the medulla would be explained as follows. It could be thought, too, that also here the adrenal cells are pressed by the extension of blood capillaries like in the cortex, and accordingly the secretory granules in the cell bodies infiltrate in the capillaries. As in the medulla, however, exist else many secretory nerve fibres directly supplying

the gland cells unlike in the cortex, so it seems to be rather right to think that the mechanism would be based upon the existence of the special nerve fibres than the extension of the capillaries.

b. Nerve cells

Some of the investigators have reported, that the nerve cells found in the medulla of the suprarenal gland show quantitatively not a little the individual fluctiation. On my studies, however, such a phenomenon seems to happen relative rarely. Besides, also in distribution they have no difinite rule, though they are seemed largely to exist in the middle part of the parenchyma or in the connective tissue along the medullar venes. The number of the nerve cells is abundant beyond my expection and they gather generally in groups from 2 to 17, as seen in Figg. 7, 8, 9, 10 and 11, although sometimes they exist lonely.

Concerning the fine features of these nerve cells none of the investigators has succeeded in staining them ideally by the silver impregnation, because of the extreme difficulty of the staining. Even the staining of the nerve cells by Stöhr could not be ideal, for, as known from his figures, not only the neurofibrils in the nerve cells are scarcely stained, but also the processes from the cell bodies are not so crarified. It is, however, very interesting and must be a great problem, that Stöhr[15] has found the special

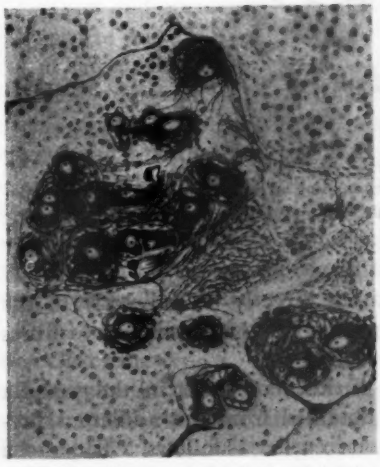


Fig. 7. Many nerve cells found in the centre part of the medulla of corpus suprarenale in adult human. Club- and ring-formed bodies are seen here and there in the neighbourhood of the nerve cells. Same staining, ×320, reduced to ½.

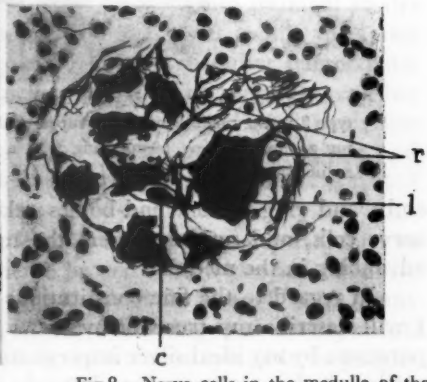
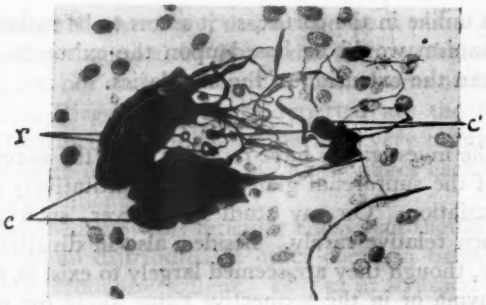


Fig 8. Nerve cells in the medulla of the suprarenal gland in adult human. l cell of Dogiel's type 1; c club-formed body; r ring-formed bodies. Same staining, ×600, reduced to ⅔.



Fif. 9. Two nerve cells of Dogiel's type 1 forming each plexus-like termination of their short process in good developed one-sided plasmodium (Stöhr). Medulla of corpus suprarenale in adult human. c two club-formed bodies terminating in contact with each nerve cell or imbedding in it; c' club bodies formed apart from the nerve cells; r ring-formed bodies. Same staining, ×600, reduced to $\frac{2}{3}$.

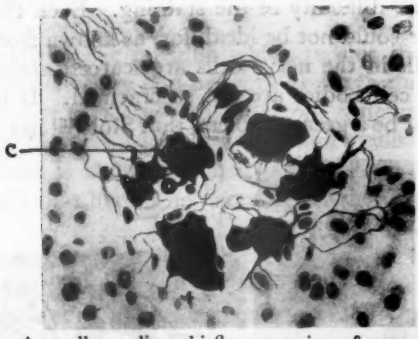


Fig. 10. A small ganglion chiefly composing of nerve cells of Dogiel's type 2 in the medulla of corpus suprarenale in adult human. c nerve cell having a short process terminating with a ring body. Same staining, ×60, reduced to $\frac{2}{3}$.

club- and ring-formed end-bodies relating directly or indirectly to the nerve cells, and supposed them the terminations of sensory nerve fibres advancing in the medulla.

In regard to the fine constitutions of the nerve cells in the medulla I will describe my trustworthy views obtained from the numerous preparations by my ideal silver impregnation in order as follows. The nuclei are largely big and round, and chiefly in the centre of the cell body, but sometimes excentric. The cells are in general mononuclear, but rarely morenuclear, for example 2–3 nuclei exist in the same cell. In the surroundings of the cell body lies the syncytial mantel cells layer or Stöhr's so-called " Nebenzellen-Plasmodium " in which the nuclei are almost one-

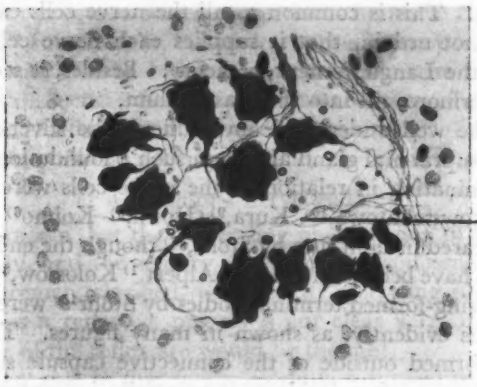


Fig. 11. A small group of nerve cells in the medulla of the suprarenal gland in adult human. t pericellular terminalreticulum; club- and ring-formed bodies are seen here and there. Same staining, ×600, reduced to ½.

layered. Besides, there is a weak connective tissue capsule which surrounds this plasmodium.

The nerve cells contain the network of finest neurofibrils and are multipolar, that is, send many processes from the surface. According to the nature and arrangement of these processes they are divided largely into two sorts of cells of Dogiel's type 1 and type 2. As a large cell pictured in Fig. 8 shows, the cells of type 1 have many short processes and largely 1–3 long processes. The formers terminate with various terminal modes after branching in the plasmodium without leaving it, while the latter leave the plasmodium and advance distantly in the medullar parenchyma to show finally for the gland cells such a supplying relationship as before described. The cells of type 2 have, as shown in Fig. 11, relatively few of short processes, all of which leave the connective tissue capsule and terminate in not so distant places from the mother cells, and largely 1–2 long processes.

Two nerve cells showed in Fig. 9 are looked upon as a special form of the cells of the type 2. All of the nerve processes show the one-sided rise and terminate in the plasmodium which developes strongly with many special nuclei, showing their complicated branching and arrangement. These special cells, however, exist also in the common sympathetic ganglia and in the intramural ganglia, for example, in those of the atrium cordis or of the intestines. Also from this point the nerve cells in the medulla should be evidently of sympathetic nature.

In the connective tissue capsule surrounding the nerve cells is formed the so-called pericellular terminalreticulum originated in the out-nerve

fibres (Fig. 11). This is common to all the nerve cells existing together and fate does not ordain, that it supplies each nerve cell separately as imaginated in the Langley's neuron theory. Besides, it seems it does not advance, furthermore, till into the plasmodium.

It would be well understood how difficult the silver-staining of the medulla of the suprarenal gland also from such a foundation, as the special end-bodies terminating in relation to the nerve cells were not shown by Pines and Narowtschatowa,[10] Kura,[9] Hoshi,[5] Kohno[6] and etc., but have been appeared in sight first by Stöhr,[15] though the end-bodies similar to them seem to have been found also by Alpert,[1] Kolossow,[8] and Kolmer.[7] Those club- or ring-formed terminal bodies by Stöhr[15] were proved also in my preparations evidently, as shown in many figures. The club bodies generally are formed outside of the connective capsule surrounding the nerve cells, however, often inside of it especially in contact with the cell body or imbedding in it (Fig. 9).

The distribution of the club bodies largely agrees with that in Stöhr's views. But, in my cases their mother fibres are represented by the short processes from the nerve cells, as shown evidently in Fig. 11. They exist here in great number and accordingly look to be specific in the medulla of the suprarenal gland, but, they are often not only in the medulla, but also for example in the atrium cordis (Seto)[16a] or in the lung (Hayashi).[3] From these reasons it is thought of unfit that Stöhr[15] supposes they will be the terminations of sensory fibres. In my thought these special terminations probably consists in making stronger the proper significance of the short processes as receptors, especially those embedding in the cell body in showing the most intimate relationship concerned the transmission of stimuli between both nerve cells.

Also in regard to the ring-formed bodies my observations almost agree with those of Stöhr. As seen in Figs. 7, 8 and 10, they exist numerously either outside of the connective capsule or inside of it just like the formers, but more or less scattered. By the way, these bodies seem to have not been found in other organs in the body unlike the club bodies. Therefore, they are thought to be the special terminal bodies existing only in the medulla of the suprarenal gland.

Stöhr supposed that these nerve rings also would be sensory endings like the club bodies. I have also felt very difficult to clarify what the stem-fibres for these bodies were, as Stöhr[15] did, because, as easily observed from the figures, nearly all of the nerve rings have no stem-fibres, but show the solitary existence. Since I thought, however, these bodies would not be sensory like the club bodies, but represented by a sort of the terminations of the short processes from the nerve cells, I have carried out on this problem my detailed examination. As a result I could finally often meet the

end of the short processes which passes into the nerve ring, as shown in Figs. 9 and 10. It is thought, therefore, that such many nerve rings having no stem-fibre with will be based upon, that their stem-fibres are largely cut off in the thin sections because of enorm thinness. In addition finally these nerve rings might be represented morphologically as nerve balls having each space like fat cells.

c. Sensory nerve ending

It is well known that the descending phenomenon of blood pressure is done reflectively, and the sensory nerve endings concerned this also have been lately extremely clarified, while on its ascending phenomenon even whether there is a reflective process is not clear yet. But the possibility of the existence of the reflex are would be thought.

I have been able to find in the medulla a typical corpusclar sensory ending, though it was only one example among many preparations (Fig. 12). It is represented by the formation of a relatively complicated glome-

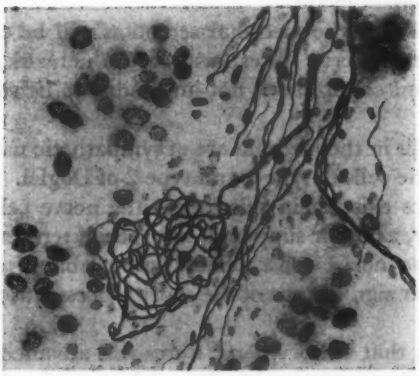


Fig. 12. A sensory glomerular termination in the medulla of the suprarenal gland in adult human. Same staining, ×600, reduced to $\frac{2}{3}$.

rular body composing of few branches suddenly divided from a probably myelinated thick fibre which show the special nuclei here and there, but without connective capsule. By the way, it is thought of physiologically very interesting, that in the medulla of the suprarenal gland can also exist sensory nerve endings though a very few.

Summary

The cortex of the suprarenal gland largely receives nerve supply neither from the plexus capsularis nor plexus medullaris, while the vegetative elements running along the blood vessels which finally pass into the

terminalreticulum by Stöhr is recognized. The gland cells, however, are not always supplied by this reticulum, but mostly rather free from its supply. Then, the mechanism of the cortical secretion will be probably based upon that the gland cells are pressed by the extension of the blood capillaries, and accordingly the intracellular granules infiltrate in the capillaries.

The medulla is favoured with nerve elements remarkably unlike the cortex. They take their rise in the nerve bundles coming from the plexus capsularis and passing through the cortex into the medulla, as well as in the sympathetic nerve cells existing in the medulla. After forming a complicated, but irregular plexus, the nerve fibres separate each other. They are more or less thick and characteristic by showing the special winding and variety of thickness. Besides, they do never terminate freely, but anastomose with each other, to form a great network. This is, of course, of vegetative nature, but morphologically differs from the common terminalreticulum remarkably. The medullar cells are supplied by this network with a contact mode.

The mechanism of the medullar secretion will be chiefly based upon those many secretory nerve fibres forming the network, although the extension of the blood capillaries is not impossible to be supposed, too, as in the cortex.

The nerve cells in the medulla are of sympathetic nature, because they are divided into the cells of type 1 and type 2 of Dogiel. Stöhr's club- and ring-formed bodies existing in relation to the nerve cells are seemed not to be such sensory, as Stöhr supposed, but special terminations of the short processes from the nerve cells. They will probably consist in making stronger the proper significance of the short processes as the recepting end-apparatus.

It is thought, that sensory nerve fibres will advance into the medulla, though very few, because sensory glomerular terminations rarely can be seen.

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Studies on the Antibiotic Substances from Actinomyces

(4th Report)

Isolation of Streptomycin from Str. olivaceus

By

Shina Kobayashi, Jiro Sato, Nakao Ishida and
(小林 しな) (佐藤 二郎) (石田 名香雄)
Yoriko Owaki
(大脇 綱子)

(From the Department of Bacteriology, Faculty of Medicine, Tohoku University, Sendai. Director: Prof. M. Kuroya)

(Received for publication September 18, 1951)

In the previous papers[1)2)] we classified 1st group of antagonistic actinomyces into several subgroups according to the results of our primary and secondary screening, and 6 non-chromogenic strains resembling *Str. griseus* were determined as streptomycin producing one by their antibacterial spectra shown by the strains as well as by the filtrates.

Now, the antibiotic produced by the one of the above 6 strains (No. 346) was identified chemically as streptomycin and moreover the taxonomic study, performed with this strain revealed that this strain was belonging to *Str. olivaceus*, not to *Str. griseus*. These data will be reported in this paper. (The chemical purification was performed in 1948, and reported in Japanese Journal of Penicillin[3)] and some taxonomic studies were made in 1949 to 1950.)

Experimental

The properties of 6 *streptomycin producing strains*

Growth ability on streptomycin containing agar and antibacterial spectra of the 6 strains (Nos. 346, 342, S-39, 304, 303 and 307) were examined and the results were shown in Table I. Against 12 test bacteria[1)], the results of cross-streaks of these strains were coinsided good with that of streptomycin itself, except No. 307. This last strain showed a somewhat different antibacterial spectrum against acid-fast organisms. It was, however, not further investigated on account of its low potency. With regards to the growth ability of No. 346 on streptomycin containing agar, some studies were made further and will be reported by Kikuchi in 8th report and by Takahashi elsewhere.

TABLE I

Primary Screening of 6 Streptomycin-Producing Streptomyces Strains

Stain No.	Growth on streptomycin containing agar				Primary screening (cross-streaks)				
					Length of inhibition zone				Activity on‡ acid-fast bacteria
	SM (μg/cc)	Growth*	SM (μg/cc)	Growth*	E. coli	SM resistant E. coli	B. subtilis	B. anthracis†	
346	200	++	400	+	13.0	0	13.0	17.4	Sm>Ph>Av
342	100	++	200	±	12.0	0	11.5	15.0	Sm>Ph>Av
S-39	100	+	200	−	8.0	0	8.2	9.5	Sm>Ph>Av
304	50	+	100	−	1.0	0	1.6	6.7	Sm>Ph>Av
303	10	+	50	−	9.0	0	11.0	15.0	Sm>Ph>Av
307	10	+	50	−	7.0	0	4.0	8.0	Av>Ph>Sm

* ++ good growth, + growth, ± few colonies, − no growth.

† Streptomycin inhibits B. anthracis stronger than B. subtilis, whereas streptothricin-like substances inhibit B. subtilis stronger than B. anthracis (according to Umezawa).

‡ Streptomycin shows the higher inhibition in order of My. smegmatis>My. phlei>My. avium (Sm>Ph>Av) in cup assay.

No .307 shows, as shown in the Table, a somewhat different antibacterial spectrum.

Antiyeast activity of the strain No. 346 also was proved by cross-streak method and "actidione" producing ability of this strain was anticipated.

Taxonomic studies

Morphological and biochemical research of the strain No. 346 was made further according to Waksman and Bergey's description. The descriptions concerning with *Str. griseus* as well as *Str. olivaceus* are given under paragraph F, p. 931 of Bergey's *manual of determinative bacteriology*, 6th edition, 1948. The most distinguished difference between both the streptomyces appears to be in the respect, that the aerial mycelium of the former is water green, while that of the latter mouse gray to light drabe. Streptomyces No. 346 appeared to resemble *Streptomyces olivaceus* more closely than *Str. griseus* in this and other many respects, as shown in Table II. In a similar way the strain No. 304 was also investigated and designated as *Str. poolensis* (Kurosawa). The utilization test of carbon sources was made according to Pridham,[4] as shown in Table III. In this respects both strains, Nos. 346 and 304 were found to be quite identical with streptomycin producing *Str. griseus*. Waksman reported many streptomycin producing strains belonging to *Str. griseus*,[5] Umezawa also with regards to

TABLE II

Characteristics of Streptomyces No. 346 Compared with Those of *Str. olivaceus* and *Str. griseus*

		Strain No. 346	*Str. olivaceus*	*Str. griseus*
Synthetic agar	Growth	Developing deep into the medium; colorless; reverse white at first, later becoming darkish.	Abundant, spreading, developing deep into the medium; color of growth yellow to olive-ocher (Rdg. 21″) reverse yellow to almost black.	Thin, spreading, developing deep into the medium, at first colorless, then turning olive-buff (Rdg. 21‴ d). This pigment may not be produced on successive transfers.
	Aerial mycelium	Thin, powdery, color white, changing to light drab, later becoming pale neutral grey to fuscous (Rdg. 13⁗ k).	Mouse-grey to light drab (Rdg. 17⁗–c).	Thick, powdery mass of a water green (Rdg. 25‴ d) color.
	Soluble pigment	None	None	None: reverse of growth brownish with age (24 days).
Ca-malate agar	Growth	Large colony, raised, wrinkled, color white; reverse faint grey.	Thin, growth penetrating to some extent into the medium, edge myceloid, color deep colonial buff (Rdg. 21″ b).	Growth thin, spreading, penetrating deep into medium; color greenish yellow with dark shade.
	Aerial mycelium	Powdery; color white to light drab, later becoming drab to mouse grey.	Thin, over large part of surface; minute droplets of water forming a silvery zone on surface of it; color violet-grey (Rdg. 59⁗) with yellowish margin.	Thin, covering all colony except a narrow edge; color approaching tea-green (Rdg. 25⁗ b).
	Soluble pigment	None	None	None
Glucose agar	Growth	Colorless at first, later becoming colonial buff (Rdg. 21″ d)—chamois (Rdg. 19″ b): reverse brown to blue almost black.	Abundant, restricted on surface and penetrating to some extent into the medium; center raised; edge entire; color deep colonial buff.	Elevated somewhat at center, radiating towards periphery; edge broken; cream-colored, with shade of orange.
	Aerial mycelium	Powdery, white to mouse grey, center plumbeous (Rdg. 49⁗ b) colored, later becoming drab-grey (Rdg. 17‴ d)—light drab (Rdg. 17⁗ b)—drab.	Covering only portions of surface; color violet grey.	Appearing only in dried-up portion of growth in fine powdery form, white colored, later turning cream-colored.

		Strain No. 346	*Str. olivaceus*	*Str. griseus*
Glucose agar	Soluble pigment	None	None in 15 days, in 35 days faint yellow.	None
Nutrient agar	Growth	Small colonies, white.	White smear, surface glistening.	Abundant, cream-colored to almost transparent.
	Aerial mycelium	None	None	Abundant, (4 days); color characteristic.
	Solbule pigment	None	None	None
Starch agar	Growth	Small colonies, color white, later becoming greyish.	Thin, spreading, yellowish green in color.	Thin, spreading transparent.
	Aerial mycelium	Powdery, smooth; color white, changing to drab-grey ~ drab, later vinaceous buff (Rdg. 17''' d) colored.	Dark grey	Grey
	Soluble pigment	None		
	Enzymatic zone	Faint	Good	12—15 mm in 15 days.
Potato plug	Growth	Much wrinkled; cream-color, changing to pinkish buff (Rdg. 17'' d)—deep olive buff (Rdg. 21''' b).	Abundant, much wrinkled, elevated, grey at first (4 days), later (8 days) turning sulfur-yellow on edge: in 30 days, the plug is nearly all destroyed (shrivelled up) by the growth of the organism.	Much wrinkled, yellowish.
	Aerial mycelium	None, later (in 18 days) poor aerial mycelium.	Abundant all over growth, light yellow with mouse grey patches.	Powdery, characteristically colored, all over growth.
	Color of plug.	Isabella color (Rdg. 19'' i)—clay-color (Rdg. 17'')—chamois (Rdg. 19'' b) an upper potion changed.	Unchanged.	Unchanged.
Gelatin (18°C)	Growth	White, changing to cream-colonial buff (Rdg. 21'' d).	Cream-colored flaky mass on bottom of liquefied portion in exposed part, close to glass, yellow.	Greenish-yellow or cream-colored with brownish tinge, developing deep into the substratum.
	Aerial mycelium	None	Thin white on yellow exposed portion of growth.	White-grey, with greenish tinge.

	Soluble pigment	None	None:	None
	Liquefaction	Rapidly	Rapid (4 cm in 35 days)	Rapid (3 cm in 35 days)
Milk (37°C)	Grow (25°)	Colonial buff ring, later becoming lily green (Rdg. 3′′′′ i) colored.	Faint pinkish growth, accompanied by hydrolysis of milk.	Cream-colored ring on surface.
	Coagulation	Rapid (3 days)	4—5 days	Rapid (2—4 days) clot formation.
	Peptonization	Rapid	Begins in 4—5 days and proceeds very rapidly so that it is all completed in 10—12 days.	Rapid (3—4 days), clearing up all the milk.
				Hydrolysis: Sometimes the tube is rapidly hydrolyzed, without previous coagulation, which is due to the strong proteolytic action.
	Change of reaction	Strongly alkaline, pH 8.0 (18 days).	Faintly to fairly alkaline.	Most alkaline.
Glucose broth	Growth	White-brownish ring and flakes on bottom of tube.	Wide, thick ring on surface in contact with glass; color sulfur-yellow; often shade of orange is produced.	Abundant mass over entire surface of liquid, of yellowish color with greenish tinge; much folded.
	Aerial mycelium	None	Thin, ash-grey, all over growth.	Powdery, of the characteristic tea-green color.
	Soluble pigment	None, later becoming brownish.	None or faint sulfur-yellow pigment.	Very faint brown beings to appear in upper portion of liquid.
Blood agar (37°C)	Growth	Small colonies, raised, wrinkled: white, later chamois (Rdg. 19′′ b) colored.		Extensive, greenish, wrinkled growth is obtained in 24 hours.
	Aerial mycelium	None		White with shade of the characteristic greenish color.
	Pigment	None		None, often faint brown.
	Haemolysis	Faint (1.7 mm zone)		Excellent, zone 1 cm, wide in 4 days.

		Strain No. 346	*Str. olivaceus*	*Str. griseus*
Peptone water	Growth	White to brown sediment.		
	Aerial mycelium	None		
	Soluble pigment	None		
Nitrate reduction		Positive	Positive	Positive
Tyrosinase		Negative	Negative	Negative
Spirals		Spirals produced on starch plate media.	None on most media.	None on most media.
Conidia		On synthetic agar, spherical or oval 0.9 to 1.1μ, 0.8 to 0.9μ×1.2 to 1.4μ.	Synthetic agar—spherical and oval, 0.9 to 1.1μ×0.9 to 2.0μ.	Synthetic agar—Rod shaped to short cylindrical, 0.8×0.8 to 1.2μ (0.8×0.8~1.5)
Habitat		Soil	Soil	Soil

its pink variants.[6] Except *Str. griseus*, however, streptomycin producing strains, such as *Str. olivaceus* were never reported. In addition chromogenic strains, Nos. K21–25 and K374 which resembled *Str. roseochromogenus* and produced streptomycin-like substance as similarly as *Str. bikiniensis* were isolated in our laboratory and with regards to antibiotic substance from K21–25 some purifications were made. The antibacterial spectrum of the filtrate of this strain was the same with that of streptomycin and crude hydrochloride obtained from it showed also a same papylographic picture with that of streptomycin, though it gave a somewhat different antibacterial spectrum by streak plate method.

Cultural condition for streptomycin production

Some cultural conditions were investigated to obtain a potent broth for purification of streptomycin using No. 346 as producing strain. The highest potencies in the several kinds of culture media were shown in

TABLE III

Utilization of Carbon Sources by Streptomycin-producing *Str. griseus* and Str. No. 346 (*Str. olivaceus*)

Compound	Strains					
	Str. olivaceus	*Str. griseus*				
	346	B_3	72	A_2	B	434
L-Xylose	+	+	+	+	+	+
L-Arabinose	(−)	(−)	(−)	−	(−)	(−)
Rhamnose	(−)	−	−	−	−	−
D-Fructose	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+
Sucrose	+	+	?	?	?	?
Maltose	+	+	+	+	+	+
Lactose	+	(+)	+	+	+	+
Raffinose	−	(−)	−	−	−	−
Inulin			(−)	(−)	(−)	(−)
D-Mannitol	+	+	+	+	+	+
D-Sorbitol	(−)	(−)	(−)	(−)	(−)	(−)
Dulcitol	−	−	−	−	−	−
DL-Inositol	(−)	−	(−)	(−)	−	−
Salicin	+	+	+	+	+	+
Sod. acetate	+	+	+	+	+	+
Sod. citrate	+	+	+	+	+	+
Sod. succinate	+	+	+	+	+	+

\+ Growth and positive utilization.
(—) Faint growth, probably no utilization.
— No growth, no utilization.
? Variable reaction, growth positive at times, negative at others.

The data about four strains (72, A_2, B, 434) were described according to Pridham and Gottlieb.

TABLE IV

Streptomycin Unit of the Culture Filtrate of *Str. Olivaceus* No. 346

	Culture media	Incubation period (days)	pH (at maximum potency)	Maximum potency (μg/cc)
Surface culture	Original Waksman's medium	12—14	7.4	28
	Thornberry's medium	10—12	7.6	153
Shaking culture	Waksman's medium with addition of 0.3 % $CaCO_3$	5— 6	7.6	126

Table IV. In the case of surface culture the culture fluids were distributed in amount of 200 cc. into each of Roux flasks, while in the case of shaking culture they were put in amount of 100 cc. into each of Sakaguchi flasks. The isolation and purification of streptomycin was made from the surface culture filtrates using Thornberry's medium (glucose 7.4%, NH_3-lactate 5.4%, KH_2PO_4 2.38%, K_2HPO_4 5.75%, $MgSO_4$ 0.98%, $ZnSO_4$ 0.0115%, $FeSO_4$ 0.0111%, $CuSO_4$ 0.0064%, $MnCl_2$ 0.0079%, and Agar 0.25%, pH 7.0), and this filtrate was found to be suitable for purification of streptomycin because it contained rather few organic materials than the others.

Recently using the strain 346, selected by the passage through streptomycin (200 μg/cc) containing agar, conditions of shaking culture were investigated in detail and a potency of 198 μg/cc was reached at 5th day of incubation using modified Vander Brook's medium containing 0.1% Teruuchi peptone. Srteptomycin Research Association of Japan[7] reported also that this strain required a specific medium for production and that under several kinds of culture media only Savage's yeast medium reached 212 μg/cc. Generally speaking, however, streptomycin producing ability of this strain was not so high.

The effect of X-ray upon the spore suspension of this strain was also examined and at the dose of 30,000 roentgen of irradiation the spore of *Str. griseus*, B_8 (U.S.A.) suspended in pH 6.5 buffer solution showed a survival rate of 2%, while this strain gave a survival rate of 100% after the same treatment.

Cross-inhibitions between many streptomycin producing strains showed also some unusual properties of this strain and will be reproted in 16th report of this series.

In many respects some characteristic peculialities different from usual streptomycin producing strains, were observed with regards to this strain.

Purification of the active substance

The purification was performed mainly according to the method of Carter *et al.*[8] The culture filtrates were combined and by the treatment with 0.5% charcoal, stirring for 15 minutes at acidic pH (pH 2.0), some impurities were removed and decolorized. Clarified filtrate, thus obtained, was further adsorbed with 2% charcoal at neutral pH, and the charcoal was washed with water and neutral 10% acetone several times. Then the carbon cake was eluted with acidic 10% acetone (pH 2.0, 1/10 volume of the filtrate) 2 times. Almost all the activity was recovered. These eluates were combined and adjusted to pH 5.0 with NaOH and occuring flocculous precipitate was removed. After the concentration *in*

vacuo active agent was precipitated with 10 volume of acetone. This precipitate was dried and redissolved in 1/10 N anhydrous HCl-methanol in order to remove inorganic salt. The active agent was precipitated again with acetone. These procedures were repeated 2 times and the crude hydrochloride was obtained in white amorphous powder assaying 160–180 u/mg.

Further chromatographic separation was made using Al_2O_3 (Japan Alum. Co. Ltd. A.P. 6) as adsorbent column according to Cartor *et al*[8] and Vander Brook *et al*.[9] The one experiment using 1.44 g. (128 u/mg) crude HCl-salt was shown in Table V. The sample was dissolved in 80%

TABLE V

An Example of Chromatographic Purification

Fraction (No.)	Volume (cc)	Potency (u/cc)	Aceton precipitate	Sakaguchi reaction	Purification
1	20	480	++	+	Partially lost, 560 u/mg, 9 mg
2	20	560	+++	++	(Sample 1)
3	25	560	+++	+++	800 u/mg, 28 mg
4	30	200	+++	++	(Sample 2)
5	50	0	−	−	
6	40	0	−	−	
7	50	0	−	−	
8	50	0	−	−	
Alumina column was eluted by acidic 80% methanol					
9	200	0	−	−	
10	200	0	−	−	

Direction: According to Cartor and others, the column was developed with 80% methanol. Alumina column: diameter 1.3 cm., length 70 cm.

methanol and adjusted to pH 6.2 with NaOH and after centrifugation supernatant was used. Al_2O_3 was treated with dil. HCl, and washed with water until no more Cl^- was proved. After resuspended in 80% methanol the column was formed. Usually, a pure hydrochloride of 500–600 u/mg was obtained with 50–60% recovery.

Purified HCl-salt (800 u/mg) thus obtained showed positive Sakaguchi and maltol test and further a crystalline reineckate was induced after recrystallization with water, which decomposed at 160–164°C.

(All the streptomycin unit assays were made by the superposing method described by Ishida *et al*.[10])

SUMMARY

Six strains of antagonistic streptomyces of our laboratory collection were determined as streptomycin producing one by their antibacterial

spectrum, and concerning with one of these strains (No. 346) designated as *Str. olivaceus* some taxonomic studies were made.

By the isolation and purification of the active principle from this culture filtrate, pure HCl-salt was obtained, showing a potency of 800 u per mg. It gave positive Sakaguchi and maltol test and a crystalline reineckate (d.p. 161–164°C).

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Studies on the Antibiotic Substances from Actinomyces

(5th Repoart)

"Secondary Screening" Test of 15 Strains of Actinomyces, Specially Antagonistic to Acid-fast Bacteria

By

Masahiko Kuroya, Nakao Ishida, Ken Katagiri,
(黒屋 政彦) (石田 名香雄) (片桐 謙)
Takehiko Shiratori and **Reiko Chida**
(白取 剛彦) (千田 禮子)

(From the Department of Bacteriology, Faculty of Medicine, Tohoku University, Sendai)

(Received for publication, September 18, 1951)

In the first step of the study on the antagonistic actinomyces, 360 active strains[1] were selected by streak plate method (so-called "primary screening") and classified into 4 groups (Table I).

TABLE I

Classification of 360 Active Strains of Actinomyces

1st group	inhibitory to gram positive, negative and acid-fast organisms	201 (56%)
2nd group	inhibitory to gram positive and acid-fast organisms	130 (36%)
3rd group	inhibitory to gram positive bacteria only	14 (4%)
4th group	inhibitory to acid-fast bacteria only	15 (4%)

For last 2 years many efforts were taken to investigate the 1st group actinomyces (inhibitory to Gram positive, Gram negative and acid fast bacteria) i.e. to divide this group of actinomyces further in details by the specificities of culture filtrates (so-called "secondary screening")[2] and to isolate the representative antibiotics of this group in crystalline forms (streptomycin[2][3] and many streptothricin-like substances[4]). Many antibiotics belonging to the 1st group inhibited acid-fast bacteria too and especially streptomycin is remarkably potent to *My. tuberculosis* (human type). Furthermore, Kelner and Morton[5] reported the isolation of an "anti-smegmatis factor" from the culture filtrate of a strain of actinomyces.

The 4th group actinomyces, however, were so chracteristic in their specific antagonistic activity against acid-fast bacteria, that it was thought

most interesting to prove their potency in their culture filtrates using several kinds of culture media and to elucidate their specific properties by comparison of their antibacterial spectra with the others according to several methods (cup-, dilution- and superposing method) and of antibacterial activities in test media of several pH.

Experimental

Methods and Materials

1) Culture media: After many experiments the following media were used in order to prove the antibacterial activities of 15 actinomyces in their culture filtrates:

For surface culture (semi-solid media containing 0.25% agar)

(1) Modified Waksman media, containing 3% glucose.

(2) Modified Waksman media, containing 2% glycerol.

For shaking culture

(1) Modified Waksman media, containing 1% glycerol.

(2) Modified Waksman media, containing 1–2% glycerol and 0.35% $CaCO_3$.

(3) Modified Waksman media, containing 1% starch.

(4) Original Waksman media, containing 1% glucose.

2) Assay method: On the 5th, 10th and 15th day in the case of surface culture, and on the 2nd, 4th and 6th day, in the case of shaking culture, samples of each culture fluid were filtered, heated at 100°C for 5 minutes and tested for their activities

(1) Dilution method: The culture filtrates were diluted at first 5 or 10 fold and then 2 fold successively with the test medium (4% glycerol broth, pH 6.5). Total volume of culture media was 5 cc. each. Their activities were tested upon *Mycobacterium phlei*, *My. smegmatis* and *My. avium* starting from 10 fold. For *Mycobacterium tuberculosis* (human type, Aoyama B strain) modified Lockemann media (pH 6.5) were used starting from 5 fold.

(2) Cup assay: Unheated culture filtrates were filled in the cup at the center on glycerol agar plates and stored in the ice-box overnight. After 24 hours, 7 test bacteria (*My. phlei*, *My. smegmatis*, *My. avium*, *My. sp.* (Clegg type), *B. anthracis*, *Staph. aureus* and *E. coli*) were streaked radially around the cup and incubated at 37°C for 2 days. The length of inhibition zone was measured in mm.

(3) One-dimensional diffusion method[6] was also used to test the activity of filtrates on Staphylococcus and E. coli. Because this method was found to be most sensitive to prove the slightest activity on these microorganisms, it was expected to be able to prove by this method the specificity of filtrates to acid-fast bacteria only.

(4) Filtrates showing activity in dilution method, were assayed again by dilution method using 4% glycerol broth of pH 6.5 and 7.5 in order to find out a suitable pH of test medium.

All the results which referred to following 3 points are shown in Tables II, III and IV.

(1) The specificity of antibiotic spectrum of culture filtrates. (dilution method and cup assay)

(2) pH of test medium, at which culture filtrate was most active.

(3) Diffusibility of antibiotics in cup assay: These observations enabled us to presume the chemical character of antibiotic substance contained in the filtrate.

Results

1) It was able to prove antibiotic activities in culture filtrates of 11 out of 15 strains, using several kinds of culture media in surface or shaking culture. Though their activity was not so powerful in general, the activity of filtrates in any assay methods corresponded almost with that of " primary screening."

TABLE II

Method of Cultivation

Strain No.	Culture method	Culture media	Incubation period (days)	pH at maximum potency
0–130	shaking	1% glycerol (0.35% $CaCO_3$)	4	7.0
364	surface	1% glycerol	12	7.6
436	surface	3% glucose	15	6.4
497	surface	2% glycerol	15	7.8
0–30				
0–101	shaking	1% glucose	4	6.0
0–128	shaking	1% glycerol	4	9.4
388	shaking	1% glycerol (0.35% $CaCO_3$)	4	7.2
309	shaking	1% glycerol	4	6.0
287	surface	2% glycerol	10	5.8
395	surface	3% glucose	10	8.2
450				
57				
27	shaking	1% glycerol	4	5.4
456				

TABLE III

Classification by the "Primary Screening"

Strain No.	Character on asparagin-glucose agar			Chromogenic (+) or not (−), on nutrient both agar	Results of streak plates (length of inhibition zone, in mm)		
	Basic mycel.	Aerial mycel.	Soluble pigment		Sm.*	Av.*	Phlei*
0-130	faint yellow	gray green	—	—	1.0	2.0	1.0
364	white	gray white	—	+	3.5	8.0	7.0
436	faint brown	white	yellow	—	1.8	2.5	2.0
497	faint yellow	gray white	—	—	0	2.3	2.0
0-30	faint yellow	red brown	—	+	5.4	3.8	3.2
0-101		white	—	—	6.7	4.5	4.0
0-128	faint yellow	yellow brown	—	+	10.5	2.5	1.0
388	white	white	—	—	1.0	1.0	0
309	white	rose	—	+	0	6.0	6.8
287	white	white	—	—	0	1.8	4.1
395	white	gray brown	—	+	0	0	7.0
450	faint yellow	gray white	—	—	0	0	2.0
57	white	gray white	—	+	0.5	0	1.0
27	faint blue	faint blue	—	+	0.5	0	0
456	white	white	—	+	0	1.5	0

* Sm; indicates *My. smegmatis*, Av; *My. avium*, and Phlei; *My. phlei*.

TABLE IV

Results of "Secondary Screening"

	Cup assay							Dilution method				One-dimensional diffusion method		Active pH of filtrate
	Sm.	Av.	Phlei	Clegg	B. anth.	Staph. aureus	E. coli	Sm.	Av.	Phlei	Human type*	Staph. aureus	E. coli	
0-130	0	0	0	0	3.2	0	0	<5	10	10	<5	—	—	alkaline
364	0	2.2	3.6	0	0	0	0	<5	10	80	<10	—	—	alkaline
436	0	0	2.0	0	2.4	0	0	<5	<5	20	<5	±	—	acidic
497	9.0	0	0	0	0	9.7	6.0	<5	10	10	5	+	+	acidic
0-30	0	0	0	0	0	0	0	<5	<5	<5	<5	—	—	
0-101	0	0	0	0	0	0	0	10	<5	10	<5	+	—	acidic
0-128	7.8	0	0	0	0	0	0	10	<5	<5	<5	—	—	alkaline
388	0	0	0	0	0	0	0	10	10	10	<5	—	—	acidic

309	0	0	0	0	0	0	0	<5	10	40	<5	—	—	acidic
287	0	0	4.1	0	5.1	16.3	0	<5	10	20	<5	+	+	neutral
395	5.3	0	2.3	0	0	0	0	<5	<5	<5	<5	+	—	
450	0	0	0	0	0	0	0	<5	<5	<5	<5	—	—	
57	0	0	0	0	0	0	0	<5	<5	<5	<5	—	—	
27	0	0	0	0	0	0	0	<5	<5	5	5	—	—	acidic
456	0	0	0	0	0	0	0	<5	<5	<5	<5	—	—	

* Indicates *My. tuberculosis* (human type).

2) Although in some cases antibiotic spectra by cup assay method coinsided good with those of "primary screening," they did not in the other. This fact has been already experienced in the screening of the 1st group actinomyces.

3) Antibiotic activity of filtrates was proved in some strains by dilution method only and could not be found out in cup assay, while some were detectable in cup assay only, but could not be proved in dilution method, suggesting different diffusibilities of each filtrate.

4) Filtrate from surface culture were sometimes higher active than that of shaking culture, so both culture methods must be tried in any case.

5) As it was expected, the antibiotic substances produced by 4th group actinomyces were almost not inhibitory to Gram-positive and negative organisms and specially active to acid-fast bacteria only. Some strains of this group, however, showed this specificity only in "primary screening", and in "secondary secrening", they were proved to be somewhat inhibitory to non acid-facst organisms too. In latter case, B. anthracis was often rather sensible than the other, as already anticipated in "primary screening".

6) Properties of active filtrates are somewhat different from each other, showing that all of them may probably produce unknown specific antibiotic substances respectively. A new antibiotic substance specially antagonistic to *My. phlei* was now isolated and crystallized by Ouchi[7] from the culture filtrate of Act. No. 364. If each of these antibiotic is purified in the future, they would certainly contribute to reveal the classification and fermentative metabolism of acid-fast bacteria.

7) It was not able to obtain more highly active filtrates to *My. tuberculosis* (human type) from this group. The 4th group of actinomyces, however, were proved to be very characteristic in the aspect of specific activity to acid-fast organisms.

SUMMARY

Fifteen strains which showed a specific activity to acid-fast bacteria only in streak plate method, were tested using several kinds of culture media on their antibiotic potency in the culture filtrates, and studied further on the nature of antibiotic substances from the point of their own antibiotic spectra, active pH and diffusibility.

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Studies on the Antibiotic Substances from Actinomyces

(6th Report)

On the Properties of 5 Kinds of Streptothricin-like Substances

By

Masahiko Kuroya, Nakao Ishida, Ken Katagiri, Jiro Konno,
(黒屋 政彦) (石田 名香雄) (片桐 謙) (今野 二郎)
Masami Kikuchi and **Ryutaro Mizuguchi**
(菊池 正美) (水口 柳太郎)

(From the Department of Bacteriology, Faculty of Medicine, Tohoku University, Sendai)

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In the research for antagonistic streptomyces, we designated the strains as 1st group actinomyces,[1] which represented antibacterial activity against several gram positive, gram negative and acid-fast bacteria, on the cross-streak test. Further, this 1st group actinomyces were classified[2] into basic and nonbasic groups according to the activity of their filtrates in alkaline or acidic test media and many basic antibiotic producing strains were obtained. Except streptomycin, almost all of these basic compounds were anticipated to be streptothricin-like substances according to their bacteriostatic nature. Further, on the account of the different diffusibility of their active agents on assay agar and several other properties, streptothricin type 1 and 2 were differentiated, the former exhibited good, whereas the latter not so good diffusibility. Now, 5 of these representative strains which appeared to produce streptothricin type 1 and 2, viz, Nos. 0–36, 0–24, 39, 259 and 120 (Seki) were selected, cultivated and chemical purifications from their filtrates were performed respectively.

All of these summarized experimental results will be reported here, and concerning with each strain further report will be made elsewhere.

Experimental

Strains tested

As streptothricin type 1 producing strains Nos. 0–36, 0–24 and 39 and as type 2 producing strains, 259 and 120 (Seki) were employed. Taxonomic studies proved that all of these strains resembled to *Str. roseochromogenus*. Only No. 24 could grow on 10 μg/cc streptomycin containing agar and became to grow abundantly on the agar containing

800 μg/cc of streptomycin after successive transfers on the agar containing gradually increasing amounts of streptomycin. After this procedure, however, there were noticed neither morphological nor biochemical changes as well as no tendency to producing streptomycin itself.

Antibacterial spectra of the strains

As shown in Table I, all 5 strains showed several properties, characteristic to streptothricin producing strains in the following respects. (1) They inhibited *Sta. aureus* (Terashima strain) stronger than *Sta. aureus* (209-P), (2) *B. subtilis* stronger than *B. anthracis*, (3) normal *E. coli* stronger than streptothricin resistant *E. coli*, while they inhibited *E. coli* in a similar degree as streptomycin resistant *E. coli*.

TABLE I

Antibacterial Spectra of the Five Strains of *Str. roseochromogenus* (Cross-streak method)

Producing strains	Inhibitory to (lengths of zones are measured in mm)										
	Sta. aureus (Terashima)	*Sta. aureus* (F.D.A 209-P)	*B. agri*	*B. anthracis*	*B. subtilis* (NRRL 558)	*E. coli*	SM* Resistant *E. coli*	0-36* Resistant *E. coli*	0-259* Resistant *E. coli*	0-20* Resistant *E. coli*	Seki* Resistant *E. coli*
No. 39	17.0	13.1	1.1	5.4	14.7	14.0	14.4	1.1	7.1	3.0	11.0
No. 24	9.0	7.0	3.0	4.0	7.0	5.0	7.0	0.4	1.0	0.8	1.4
No. 39	21.0	20.4	6.4	9.1	17.5	14.9	15.8	3.8	6.2	2.0	8.8
No. 259	12.0	9.0	7.0	7.2	10.9	8.4	11.0	8.7	8.4	5.1	4.0
No. 120 (Seki)	13.2	12.8	7.2	8.9	12.4	11.2	11.4	8.7	9.1	5.1	0

* Means *E. coli* resistant against streptomycin and against final substances obtained from the cultures of Nos. 0-36, 259, 0-20 and 120 (Seki) respectively.

In this antibacterial spectrum, however, it was scarcely possible to differentiate both streptothricin types 1 and 2 producing strains from one another. There are, however, inconformities between the antibacterial spectra of these strains and those of their final products, and will be discussed later.

Cultural condition

All of these strains were cultivated using modified Vander Brook's medium (added with 0.1 to 1% peptone instead of curbay B. G.) in shaking condition at 28°C.

Purification of the active substance

After the adjustment of the filtrate to pH 3.5, mycelium was removed

by filtration and the active agent was further purified by adsorption to and elution from carbon as already described concerning with streptomycin and streptothricin.[8] Being, treated with 0.5% charcoal and stirred for 15 minuted at acidic pH (pH 2.0), the filtrate was freed from some impurities and decolorized. Clarified filtrate, thus obtained, was further adsorbed with 1 to 2% charcoal at neutral pH, and the charcoal was washed with water and neutral 10% acetone several times. The carbon cake was then eluted with acidic 10% acetone (pH 2.0, 1/10 to 1/20 volume of the filtrate) 2 or 3 times. Almost all the activity was recovered in such a manner. These eluates were combined and adjusted to pH 5.0 with NaOH and occuring flocculous precipitate was removed. After the concentration of the solution *in vacuo* at pH 5.0 the residue was desiccated on P_2O_5, and redissolved in 1/10 N anhydrous HCl-methanol in order to remove inorganic salts. The active agent was precipitated with 10 vol. of acetone. This final procedure was repeated 2 times and the crude hydrochloride was obtained in a white amorphous powder.

The crude hydrochloride was purified by the chromatographic method using Al_2O_3 as adsorbent and further precipitated with methylorange. The induced crystalline helianthate was converted again into the hydrochloride.

In this purification course noticeable points are as follows:

1) The selection of culture media is an important problem.

If the medium rich in organic materials such as Waksman's was used, crude hydrochloride was obtained in recovery of 1 mg/cc from the broth with contamination of some brownish pigments and other impurities, which was rather difficult to be removed. When Vander Brook's semi-synthetic medium which was composed chiefly of inorganic materials was used it was easier to obtain a purified hydrochloride in much more amounts and to induce them into crystalline reineckate immediately.

2) The decolorization and removal of impurities using charcoal at acidic pH must be carried out precautiously, and if more charcoal was used some losses of activity were unavoidable. In order to obtain a highly purified preparation, however, such a procedure was necessary to be taken.

3) The elution from charcoal was usually made with 10% acetone or sometimes with 80% methanol at acidic pH. Concerning with the above antibiotics, almost all of the activities were recovered by this procedure, except No. 39 which showed always about 60% recovery, even if strongly acidic acetone was used.

4) The procedure of chromatographic separation was conducted according to Cartor's description,[8] using Al_2O_3 (Japan Alum. Co. Ltd. A.P.6) as adsorbent, and 20 to 60% recovery was obtained, and the ac-

tivity of purified hydrochloride, thus obtained, reached about 2 to 8 times of starting material.

5) For the recrystallization of helianthate 50 to 100% methanol was the most suitable solvent. The helianthate was slightly soluble in ethanol too, whereas hardly soluble in propyl-, isopropyl-, butyl-, amyl-alcohol, chloroform and ether.

The biological and chemical nature of the purified antibiotics

Concerning with these 5 kinds of purified hydrochloride, which were able to be induced to crystalline reineckates, some biological and chemical natures were compared with those of pure streptomycin (Merck) and with each other. All these results are shown in Table II.

Antibacterial properties of these compounds were tested both with cup and dilution assay method. Cup assay was conducted on nutrient agar plate for common bacteria and on 4% glycerol nutrient agar for acid-fast bacteria. A penicillinder was set at the center of each plate, being filled with each of 1 mg/cc hydrochloride solutions. They were allowed to stay overnight in an ice box. Concerning with the substance No. 259, the agar plate was stored for 48 hrs. for its low diffusibility. Next morning all of the test organisms, including normal *E. coli*, streptomycin-, No. 0–36-, No. 259-, No. 0–20 (ST 1)-, and No. 120-resistant strains of *E. coli*, were streaked radially around the cup.

The resistant *E. coli* strains toward streptomycin and each of 4 streptothricin-like substances respectively were obtained by the successive transplantations through the broth, containing highest concentrations of each of corresponding antibiotics, which permit the growth of *E. coli*.

There was seen, however, a remarkable difference between the acquired resistances toward 4 streptothricins and that toward streptomycin. A strain of *E. coli*, which was inhibited initially in a concentration of 0.6 μg/cc of streptomycin became resistant toward a concentration of 65 mg/cc of the same (24,000 fold compared with initial resistance) by the procedures mentioned above and it seemed to be possible to elevate the resistance further to a higher degree. In the case of streptothricines, however, it was not so easy to elevate the resistance of the same strain of *E. coli*, viz., the resistance toward No. 36–substance was brought up to the height of 80 fold (0.5 mg/cc) of initial resistance, those toward No. 259-, No. 0–20- and No. 120-substances were elevated to the heights of 80 (0.1 mg/cc), of 200 (0.5 mg/cc) and of 200 (0.25 mg/cc) fold respectively by the same techniques and they were coming to a stationary condition in these degrees of resistance, because it was no more possible to elevate their resistance by repeating the transfers.

The results of the antibacterial cup assays were summarized as follows:

TABLE II

Biological Properties of Streptomycin and Five Streptothricin-like Substances

HCl salt of antibiotic substances	Antibacterial spectra in cup assays (Inhibition zones are measured in mm)											Dilution unit (×1000)		
	Gram-positive organisms					Gram-negative organisms						*Sta. aureus*		*E. coli*
	Sta. aureus		*B. agri*	*B. anthracis*	*B. subtilis*	*E. coli*	SM_R *E. coli*	0–36R* *E. coli*	0–259R* *E. coli*	0–20R* *E. coli*	120R* *E. coli*	Terashima	209–p.	
	Terashima	F.D.A. 209–p.												
Streptomycin	19.4	19.3	0	22.6	15.6	18.1	0	3.7	9.6	8.0	11.0	1600	1600	160
Basic antibiotic No. 36	14.7	13.8	0	4.4	15.2	14.9	15.0	0	0	0	0	80	80	16
Basic antibiotic No. 24	11.4	10.8	0	6.4	15.4	10.9	10.0	0	0	0	0	40	40	16
Basic antibiotic No. 39	13.1	13.2	2.8	5.2	13.8	13.4	14.0	0	0	0	0	160	160	16
Basic antibiotic No. 259	11.9	11.7	6.0	7.2	11.0	11.8	11.6	0	0	0	0	2000	1000	80
Basic antibiotic No. 120 (Seki)	7.2	6.8	2.8	3.1	6.1	6.1	6.1	0	0	0	0	2000	1000	80

1) All 5 kinds of substances inhibited *B. subtilis* more strongly than *B. anthracis* on the agar plate, while streptomycin inhibited the latter stronger than the former. This relationship was useful to divide these antibiotics into streptomycin and streptothricin-like groups, besides the use of drug-resistant bacteria. Streptomycin resistant *E. coli* was only resistant to streptomycin itself and rather more susceptible to the 5 substances than normal *E. coli*. On the contrary, the resistant *E. coli* to each of 4 streptothricins respectively were resistant to all of these 5 substances and to streptomycin too, even if the resistance toward the latter was only in a limited extent.

2) *B. agri*, confirmed by Prof. Hosoya to be susceptible toward streptothricins and not susceptible to streptomycin, like Bodenheim's organism, was not inhibited by 2 out of 5 substance belonging to streptothricin type 1 (Nos. 36 and 24), whereas it was somewhat susceptible against other 3 kinds of streptothricins (39, 259 and 120).

Using *B. agri* as test organism, therefore, it was possible to differentiate streptothricin type 1 from type 2, while it was not able to distinguish streptomycin from streptothricin type 1. It was revealed further, that either streptothricin type 1 or type 2 producing strains inhibited *B. agri*, though the activity of the former was rather lower than the latter as shown in Table I. In this respects it was able to find a discomformity between both antibacterial spectra, one produced by cross streak test and the other showed by purified antibiotics. Another discomformity was found in the respect, that all streptothricin producing strains could inhibit all of the streptothricin resistant *E. coli* strains more or less, whereas none of the purified streptothricins was able to do so. With regards to these repects, the report will be made elsewhere.

3) When these cup assay titers were compared with those of dilution assay titers, both streptothricin type 2 substances (Nos. 259 and 120) appeared to be low potent due to their low diffusibilities.

Two-fold serial dilution assay was performed using less nutrient broth (meat extract and peptone 0.5% each without NaCl, pH 7.5), against *Sta. aureus* Terashima, *Sta. aureus* 209–P, and *E. coli*. Twenty four hrs. broth cultures of these bactiera were used as the inoculum, 0.05 cc of which being added to 5 cc of assay broth, and cultivated at 37°C for 18 hrs. These data coinsided good with that of culture filtrates (as described in 3rd report[2]), and this fact indicates that the chief antibiotics contained in the filtrates were extracted and purified to these hydrochlorides.

4) Streptothricin type 1 showed the same Terashima: *E. coli* unit ratio and Terashima: F.D.A. 209–P unit ratio with those of streptomycin, while streptothricin type 2 showed remarkably higher ratios in both cases.

5) The toxicity test was carried out using the mice weighing 15 g in average. Each required amount of the pure hydrochloride was dissolved in 0.3 cc physiological saline and injected both intravenously and intramusculary. Concerning with some antibiotics, maximal tolerated dosage was not determined. However, by the injection of 1/10 of lethal dosis as indicated in the Table III no toxicity was shown.

The lowest toxicity was shown by the antibiotic No. 36 and in this respect it was more preferable than streptomycin. The other substances showed a so-called "delayed toxicity" and some lung-, and liver hemorrhage on autopsy, both of which were characteristic properties of streptothricin groups.

6) All of these crystalline reineckates and helianthates were obtained in a platelet form, in contrast with streptomycin, which obtained in needles. The decomposition points of these crystals were found to be in the vicinities of that of streptothricin, whereas the reineckate of the antibiotic No. 36 showed a characteristic low decomposition point (114°C). On the other hand the antibiotic No. 39 resembled most closely Waksman's streptothricin,[4] (reineckate: d.p. 192°–194°C, helianthate d.p. 225°–230°C).

7) Some chemical reactions, using 0.25% aqueous solution of each antibiotics, were examined. Streptomycin is characteristic in positive Sakaguchi and maltol tests while 5 streptothricin substances were negative in them. Elson-Morgan's glucosamine test was positive both, in these 5 substances and streptomycin, so no unique reaction especially positive in these 5 substances were not found yet.

Summary

1) Five kinds of basic antagonistic substances, which resembled streptothricin in nature, were obtained in a form of hydrochloride, crystalline helianthate and reineckate, and the identification between these substances was performed.

2) All of these substances had rather lower potencies than streptomycin, in a dilution titer against *E. coli*, while the substances No. 259 and No. 120 (Seki) were higher inhibitory against *Staphylococcus* (Terashima strain) than streptomycin.

3) Six substances, including streptomycin, were arranged in the order of increasing toxicities as follows: —

36, streptomycin, 120 (Seki), 24, 39, and 259. The substance No. 36 was able to be injected both, intravenously and intramuscularly into mice in an amount of 20 mg without showing any toxic signs. In this respect, the substance No. 36 has advantages over streptomycin.

4) The decomposition points of helianthates or reineckates of 4 streptothricin-like substances were found to be in the vicinities of that of

TABLE III

Toxicities and Chemical Properties of Streptomycin and Five Streptothricin-like Substances

HCl salt of antibiotic substances	Toxicity	D.p. of crystals		Chemical reactions of HCl-salt (10 mg/40 cc in distilled water)						
		Helianthate	Reineckate	Naphto-resorcin (Tellens)	Fehling	Gluco-samine	Maltol	Biuret	Ninhydrin	Sakaguchi
Streptomycin	intraven. 5 mg dead intramus. 20 mg alive	220–221°	161–163°	+	+	+	+	−	−	+
Basic antibiotic No. 36	intraven. 20 mg alive intramus. 20 mg alive	211–216°	114°	+	+	+	−	−	−	−
Basic antibiotic No. 24	intraven. 5 mg dead (12 days)	275–285°	185–201°	+	+	+	−	−	−	−
Basic antibiotic No. 39	intraven. 5 mg dead (6 days)	225–230°	198–201°	+	+	+	−	−	−	−
Basic antibiotic No. 259	intraven. 1.5 mg dead (6 days)	244°	193–196°	+	+	+	−	−	−	−
Basic antibiotic No. 120 (Seki)	intraven. 10 mg alive	224–230°	184–185°	+	+	+	−	−	−	−

streptothricin except No. 36 (Roseomycin). In reference to the other biological and chemical properties, however, the 5 substances seem quite different from streptomycin and from each other.

5) Maltol and Sakaguchi reactions were found to be positive in streptomycin alone, while glucosamine and other sugar reactions were positive in both streptomycin and streptothricin-like 5 substances. The chemical reactions positive in streptothricin group only, were not found yet.

6) A strain of *E. coli*, which was made resistant to the hydrochlorides of 4 substances (36, 20, 259, and 120) respectively was resistant to each of 5 substances and somewhat less resistant to streptomycin too, while the same strain of *E. coli*, which was made resistant to streptomycin, was not resistant to each of 5 substances. In this respect it seems very probable, that each of 5 substances resembles closely in their mechanisms of the antibiotic functions.

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Biochemical Studies on Carbohydrates

CLI. On Paper Partition Chromatography of Sugars in Particular of Hexosamines and Hexuronic Acids

By

Hajime Masamune and **Masahiro Maki**
(正宗 一) (眞木 正博)

(From the Medico-chemical Institute, Tohoku University, Sendai)

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Various solvents and developers were again scrutinized for paper partition chromatography of sugars and the following findings looked most noteworthy.

1) Plotting of charts like Figs. 1 and 2 is advisable to pick up an appropriate solvent for separation of a certain couple of sugars from each other.

2) As the figures in Tables I and III indicate and as is already mentioned by authors, Rf values of sugars vary from one mixed solvent to another and even the relative positions of sugars are reversed, though rare. The rise of temperature generally increases the Rf values of a sugar (Tables IV and V) and sometimes inverts the relative positions of sugars by one and the same solvent. Rf values of individual sugars are altered further by change of the distance run, moisture content of air, purity of solvents, quality of paper strips and else so that it is indispensable to simultaneously develop a reference run employing a part of a big sheet of paper neighbouring to the one for the main.

3) The aniline-phtharic acid mixture (Partridge[1]) and a trichloroacetic acid solution in aniline proved much superior to $AgNO_3$-NH_3 (Partridge[2]) and alkaline potassium permanganate (Pascu, Mora & Kent[3]) as a developer, because of the different colorations of different kinds of sugar and of giving a plain back-ground.

4) When run as sulfate, glucosamine and chondrosamine showed different velocities by a five component solvent *n*-butylacetate-acetic acid-butanol-methanol-water (3:2:2:1:1). The spot of the glucosamine salt tails thereby as that of glucosamine hydrochloride, due to partial liberation of free glucosamine, but it does not impair the identification.

5) Contrary to the description of Werner and Odin,[4] pyridine-amylalcohol-water (7:7:6), one of their solvents, served well to separate

not only the mixture of glucuronic and galacturonic acid but also that of those hexuronic acids and mannuronic acid. Glucuronic acid gave a spot of its lactone besides. This, however, ran much quicker than glucuronic acid itself and other hexuronic acids.

Experimental

Method

Technique. Yosizawa's technique[5] was applied, by employing the aniline-phthalic acid solution in butanol saturated with water of Partridge[1] (0.93 g. aniline is mixed with 1.66 g. phthalic acid dissolved in 100 cc. of water-saturated butanol) or a 2.5% trichloroacetic acid solution in aniline (w/v) as a developer instead of $AgNO_3$-ammonia or Elson-Morgan reagent (Paper strips, 3 or 2.5 cm. width; 0.02 cc. of 1% sugars was added as a circular spot of 0.8 cm. diameter; quantity of solvents for the run 7.0 cc. or 14 cc.).

The solvents used:—1) *n*-butylacetate-pyridine-water (5 : 3 : 1) of Yosizawa, 2) *n*-butylacetate-acetic acid-methanol-water (3:2:0.5:0.5), 3) *n*-butylacetate-acetic acid-butanol-methanol-water (3 : 2 : 2 : 1 : 1), 4) *n*-butylacetate-acetic acid-butanol-methanol-water (3: 2: 0.5: 2: 0.5), 5) *n*-butylacetate-acetic acid-water (3: 2: 1) of Yosizawa, 6) amylalcohol-pyridine-water (7: 7: 6) of Werner and Odin, and 7) benzylalcohol-acetic acid-water (3: 3: 1).

Reference Sugars

1) Rhamnose Takeda (extra pure).
2) Mannose Takeda („ „).
3) Xylose Takeda („ „).
4) Arabinose Takeda („ „).
5) Galactose E. Merck.
6) Glucose E. Merck.
7) Glucosamine hydrochloride.
8) Free glucosamine prepared according to Breuer.[6]
9) Glucosamine sulfate (amorphous). 90 mg. of free glucosamine were dissolved in a little water and after addition of 0.6 cc. of 1 *N* H_2SO_4 which was slightly more than the equivalent quantity, precipitated with an alcohol-ether (1: 1 by volume) mixture. The deposit was centrifuged and washed thoroughly with alcohol and further with ether and placed in a desiccator. 70 mg. was the product. N 6.0% (Calc. 6.16%); ash 0.0%.
10) Chondrosamine hydrochloride.
11) Chondrosamine sulfate (amorphous). To 0.2 g. of chondrosamine hydrochloride in a little water were added 1.5 cc. of 1 *N* H_2SO_4 at first and then, after a while, 10 cc. of an alcohol-ether (1: 1 by volume) mixture.

The deposit was washed with abs. alcohol and after drying, again treated with sulfuric acid similar to above to completely substitute H_2SO_4 for HCl. The deposit here was washed with alcohol and ether and dried. N 6.2% (Calc. 6.16%), ash 0.0%.

12) Glucuronic acid. Prepared from menthol-glucuronic acid.[7]

13) α-Galacturonic acid. It was separated at first as barium salt from beet pectin. A watery solution of this salt was decolorized with charcoal, liberated from barium with sulfuric acid and distilled in vacuo to a thick solution. On standing in an ice-chest, crystalline deposits in thin needles came out which were washed with alcohol. F. P. 108–110°C, decomposition point 158–160°C; crystal water 8.6% (Calc. for 1 mole crystal water, 8.47%); optical rotation of the anhydrous substance in water: $[\alpha]_D^2=\frac{+2.39\times 100}{2.29\times 1}=+104.4°$ (5 minutes after dissolution) $\rightarrow \frac{+1.29\times 100}{2.29\times 1}$ $=+56.3°$ (at equilibrium). [Cf. Ehrlich and Schubert[8]: F. P. 110°C, decomp. 156–159°C, $[\alpha]_D^{20}=+101.7°$ (initial) $\rightarrow +55.6°$ (at equilibrium)]

14) Barium mannuronate. Free alginic acid was liberated from sodium alginate according to Nelson and Cretcher[9] and 3.2 g. of barium mannuronate were obtained after Link and Nedden[10] from 10 g. of the acid polysaccharide. Molisch negative, Goldschmiedt positive.

Ash ($BaCO_3$)

32.5 mg. substance, 12.3 mg. ash.

$Ba(C_6H_9C_7)_2$. Found, 37.8%; calc., 37.7%.

To the chromatography, a solution of this salt was applied after eliminating barium with an equivalent quantity of H_2SO_4 and diluting to 1% with respect to the free sugar.

15) Mucosin sulfate (amorphous). To 100 mg. of free mucosin, prepared as had been by Isikawa[11], were added 3 cc. of 0.1 N H_2SO_4. A trace of an insoluble part was centrifuged off and the supernatant fluid precipitated with an alcohol-ether (1 : 1 by volume) mixture and after washing with alcohol and ether, dried. Yield 75 mg. N 3.3% (Calc., 3.4%); ash 3.0%. Chloride test negative; Molisch faintly positive; Goldschmiedt and Neuberg-Saneyoshi distinctly positive.

16) Chondrosinsulfate (amorphous). 100 mg. of chondrosinethylester hydrochloride (Hebting) were taken up in 2 cc. of water, the saturated $Ba(OH)_2$ added to a pH of 9.0 and the mixture stood in an ice chest for several hours. The pH lowered was raised to the original by addition of additional baryta and the mixture was stood cooled overnight. Thereafter barium was carried down with an equivalent quantity of H_2SO_4 and, in addition, 3 cc. of 0.1 N H_2SO_4 were added. The centrifuged supernatant fluid was evaporated in vacuo to about 2 cc., precipitated with alcohol and washed with alcohol. The substance dried was reprecipitatep

from 2 cc. of 0.1 *N* H_2SO_4 followed by washing with alcohol and ether and dried. N 3.5%, ash 0.0%. Chloride and Molisch test negative; Goldschmiedt strongly positive.

Results

1. When the temperature was maintained at 20°C and solvent fronts were run 25 cm.

The distances travelled are shown in Table I, together with the respective Rf values in parentheses which are also plotted in Fig. 1.

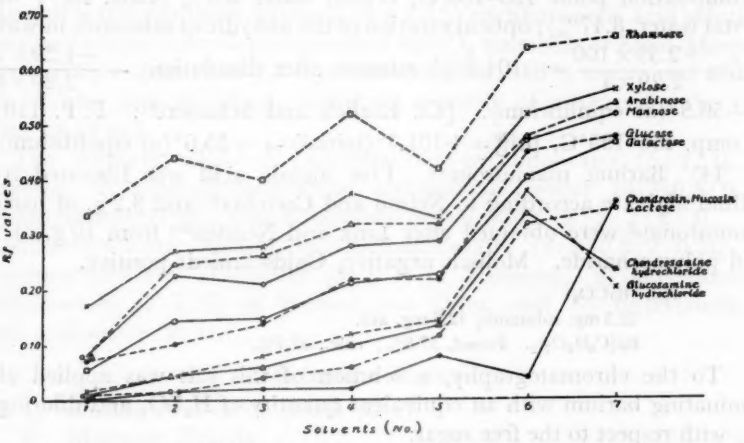

Fig. 1. Rf values of sugars at 20°C varying according to the solvent (Table I) are expressed in a chart.

In Table II, the Rf values obtained with Solvents 1, 5 and 6 (Table I) and those by Yosizawa are brought into comparison. Significant differences are noticed between the corresponding two values, when Solvent 1 was used, despite of the apparently same technique.

2. When the temperature was maintained at 25°C

Solvents 3 and 4 were run 45 cm., and Solvents 6 and 7 40 cm. Table III and Fig. 2 illustrate the results.

Higher temperatures give higher Rf values generally, as will be demonstrated in Tables IV and V. Therefore, that most of the Rf values at 25°C are smaller than the corresponding values at 20°C (in Table I) is thought due to the diminution of the Rf values when sugars are run longer.

3. Separation of hexosamines

Again Solvents 3, 5, 6 and 7 were examined, taking not only the

TABLE I

Distances and Rf Values (in parentheses) of Sugars when the Solvent Fronts were Run 25 cm. at 20°C

Paper strips 28 cm. × 3 cm.; solvents 7.0 cc. Developed with aniline-phthalic acid reagent of Partridge.

Solvent No.	Composition		Duration of test hrs.	Movement of the center of sugar spots from the starting point (cm.)										
				Rhamnose	Xylose	Arabinose	Mannose	Glucose	Galactose	Lactose	Glucosamine hydrochloride*	Chondrosamine hydrochloride*	Mucosin sulfate	Chondrosin sulfate
1	*n*-Butylacetate	5	3	8.4 (0.336)	4.3 (0.172)	2.0 (0.08)	2.0 (0.08)	1.8 (0.072)	1.4 (0.056)	0.2 (0.008)	0.4 (0.016)	0.2 (0.008)	0.0 (0.0)	0.0 (0.0)
	Pyridine	3												
	Water	1												
2	*n*-Butylacetate	3	$4\frac{1}{6}$	11.0 (0.44)	6.8 (0.272)	6.2 (0.248)	5.7 (0.228)	2.6 (0.104)	3.6 (0.144)	0.4 (0.016)	1.1 (0.044)	1.1 (0.044)	0.4 (0.016)	0.4 (0.016)
	Acetic acid	2												
	Methanol	0.5												
	Water	0.5												
3	*n*-Butylacetate	3	$4\frac{1}{2}$	10.0 (0.40)	7.0 (0.28)	6.6 (0.264)	5.3 (0.212)	3.5 (0.14)	3.7 (0.148)	1.0 (0.04)	2.0 (0.08)	1.5 (0.06)	0.5 (0.02)	0.5 (0.02)
	Acetic acid	2												
	Butanol	2												
	Methanol	1												
	Water	1												
4	*n*-Butylacetate	3	$4\frac{1}{3}$	13.0 (0.52)	9.4 (0.376)	8.0 (0.32)	7.0 (0.28)	5.5 (0.22)	5.3 (0.212)	1.5 (0.06)	3.0 (0.12)	2.5 (0.10)	0.5 (0.02)	0.5 (0.02)
	Acetic acid	2												
	Butanol	0.5												
	Methanol	2												
	Water	0.5												
5	*n*-Butylacetate	3	$4\frac{1}{8}$	10.5 (0.42)	8.3 (0.332)	8.0 (0.32)	7.2 (0.288)	5.5 (0.22)	5.7 (0.228)	2.4 (0.096)	3.6 (0.144)	3.4 (0.136)	2.0 (0.08)	2.0 (0.08)
	Acetic acid	2												
	Water	1												
6	Amylalcohol	7	$8\frac{1}{8}$	16.0 (0.64)	13.0 (0.52)	12.0 (0.48)	11.8 (0.472)	11.3 (0.452)	10.1 (0.404)	8.0 (0.32)	9.5 (0.380)	8.5 (0.34)	1.0 (0.04)	1.0 (0.04)
	Pyridine	7												
	Water	6												
7	Benzylalcohol	3	7	16.5 (0.66)	14.1 (0.564)	13.5 (0.54)	13.0 (0.52)	12.0 (0.48)	11.7 (0.468)	8.7 (0.348)	5.1 (0.204)	5.9 (0.236)	9.0 (0.36)	9.0 (0.36)
	Acetic acid	3												
	Water	1												

* The spots were mostly tailed with free hexosamine, which is not taken into account for reading the distances.

TABLE II

Comparison of Rf Values of Sugars Obtained under Similar Conditions by Yosizawa and by the Writers

Paper strips 28 cm. × 3 cm.; solvents 7.0 cc. Solvent fronts run 25 cm. Temperature 20°C.
Developed with aniline-phthalic acid reagent of Partridge.

Solvents No.	Composition	Author	Duration of test hrs.	Movement of the sugar spots from the starting point (cm.)								
				Rhamnose	Xylose	Arabinose	Mannose	Glucose	Galactose	Lactose	Glucosamine hydrochloride*	Chondrosamine hydrochloride*
1	*n*-Butylacetate 5 Pyridine 3 Water 1	Yosizawa	3	0.39	0.304	0.224	0.216	0.16	0.124	0.06	0.024	0.016
		The writers	3	0.336	0.172	0.08	0.08	0.072	0.056	0.008	0.016	0.008
5	*n*-Butylacetate 3 Acetic acid 2 Water 1	Yosizawa	5†	0.44	0.33	0.32	0.29	0.25	0.256	0.136	0.13	0.07
		The writers	$4\frac{1}{2}$†	0.42	0.332	0.32	0.288	0.22	0.228	0.096	0.144	0.136
6	Amylalcohol 7 Pyridine 7 Water 6	Yosizawa	$8\frac{1}{2}$	0.63	0.556	0.484	0.51	0.464	0.42	0.32	0.296	0.29
		The writers	$8\frac{1}{2}$	0.64	0.52	0.48	0.472	0.452	0.404	0.32	0.38	0.34

* Signifies the same as in Table I.

† The durations of test did not coincide with each other.

TABLE III

Distances Travelled and Rf Values (in parentheses) of Sugars Run at 25°C

Paper strips 60 cm. × 2.5 cm.; solvents 14 cc. Fronts of Solvents 3 and 4 were run 45 cm. and those of Solvents 6 and 7 40 cm. Spots were developed with aniline-phthalic acid reagent of Partridge.

Solvent No.	Composition		Duration of test hrs.	Movement of center of sugar spots from the starting point (cm.)									
				Rhamnose	Xylose	Arabinose	Mannose	Glucose	Galactose	Glucuronic acid†	Galacturonic acid	Glucosamine hydrochloride*	Chondrosamine hydrochloride*
3	*n*-Butylacetate	3	19½	17.3 (0.384)	11.4 (0.253)	9.9 (0.22)	8.7 (0.189)	6.0 (0.133)	6.1 (0.136)	4.7 (0.104)	2.7 (0.06)	4.0 (0.089)	3.5 (0.078)
	Acetic acid	2											
	Butanol	2											
	Methanol	1											
	Water	1											
4	*n*-Butylacetate	3	15½	19.0 (0.426)	13.5 (0.3)	10.6 (0.236)	10.4 (0.231)	9.0 (0.2)	7.4 (0.164)	2.2 (0.049)	1.8 (0.04)	4.3 (0.096)	4.0 (0.089)
	Acetic acid	2											
	Butanol	0.5											
	Methanol	2											
	Water	0.5											
6	Amylalcohol	7	23	22.7 (0.568)	18.8 (0.47)	16.9 (0.423)	16.8 (0.42)	15.1 (0.378)	13.3 (0.336)	6.0 (0.15)	0.7 (0.018)	11.0 (0.275)	10.3 (0.258)
	Pyridine	7											
	Water	6											
7	Benzylalcohol	3	22½	25.6 (0.64)	21.3 (0.543)	22.0 (0.55)	19.6 (0.49)	18.7 (0.468)	17.8 (0.445)	12.0 (0.3)	11.7 (0.298)	8.1 (0.205)	9.5 (0.238)
	Acetic acid	3											
	Water	1											

* Signify the same as in Table I. † It gives a spot of glucuronolactone besides.

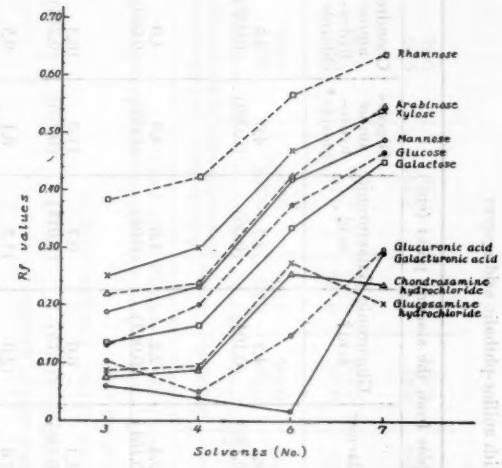

Fig. 2. Rf values of sugars by different solvents at 25°C (Table III) are expressed in a chart.

hydrochlorides but also the sulfates of glucosamine and chondrosamine. Free glucosamine was also run besides.

The fronts of Solvents 3 and 5 were moved 45 cm. and those of Solvents 6 and 7 40 cm.

a) The effect of temperature. At 20, 25 and 30°C were run glucosamine- and chondrosamine-hydrochloride. As is illustrated in Table IV (Six of the figures are reprinted from Table III), Rf values of the hexosamine hydrochlorides were found to increase with the rise of temperature.

b) From the same table it looked that the hydrochlorides of the hexosamines can be separated quite well from each other by Solvent 7 at 20 and 25°C and the sulfates by Solvents 3 and 5 (30°C is most suitable here). When Solvent 6 was used, the spots of the hydrochlorides or those of the sulfates fell on much nearer positions. On the other hand, the spots of the glucosamine salts tailed till the positions corresponding to free glucosamine, which was most striking when Solvent 7 was used. Since the hydrochloride of chondrosamine travels farther by Solvent 7 than that of glucosamine, their spots join together through the tail of the latter specimen. Those of the chondrosamine salts tailed short or did not at all as in case the sulfate was run by Solvents 3 and 5. In short, it is recommendable for the aim in view to apply Solvents 3 and 5 to the sulfates. Fig. 3 demonstrates diagrammatically the chromatograms obtained with the former solvent at 30°C.

TABLE IV

Distances Travelled and Rf Values (in parentheses) of Hydrochlorides and Sulfates of Glucosamine and of Chondrosamine and Free Glucosamine when Run at 20, 25 and 30°C by Solvents 3, 5, 6 and 7

Paper strips 60 cm. × 2.5 cm.; solvents 14 cc. Developed with ninhydrin.

Solvent No.	Solvent Composition		Solvent front cm.	Temperature °C	Duration of test hrs.	Movement of center of sugar spots (cm.)				
						Glucosamine hydrochloride	Chondrosamine hydrochloride	Glucosamine sulfate	Chondrosamine sulfate	Free glucosamine
3	*n*-Butylacetate	3	45	20	20	2.8 (0.062)	2.5 (0.056)			
	Acetic acid	2		25	19½	4.0 (0.089)	3.5 (0.078)			
	Butanol	2		30	19	6.3 (0.196)	5.5 (0.122)	2.0 (0.044)	0.4* (0.009)	8.8 (0.196)
	Methanol	1								
	Water	1								
5	*n*-Butylacetate	3	45	20	24½	5.0 (0.111)	4.6 (0.102)			
	Acetic acid	2		25	24	5.8 (0.129)	5.1 (0.119)			
	Water	1		30	23	6.5 (0.144)	6.0 (0.133)	1.9 (0.042)	0.5* (0.011)	12.5 (0.278)
6	Amylalcohol	7	40	20	23½	10.5 (0.263)	10.0 (0.25)			
	Pyridine	7		25	23	11.0 (0.275)	10.3 (0.258)			
	Water	6		30	22½	12.0 (0.3)	11.7 (0.292)	11.6 (0.29)	11.3 (0.283)	12.0 (0.3)
7	Benzylalcohol	3	40	20	23	7.5 (0.188)	8.7 (0.218)			
	Acetic acid	3		25	22½	8.1 (0.205)	9.5 (0.238)			
	Water	1		30	21½	9.5 (0.238)	9.8 (0.245)	6.0 (0.15)	6.6 (0.165)	18.5 (0.463)

* No tailing.

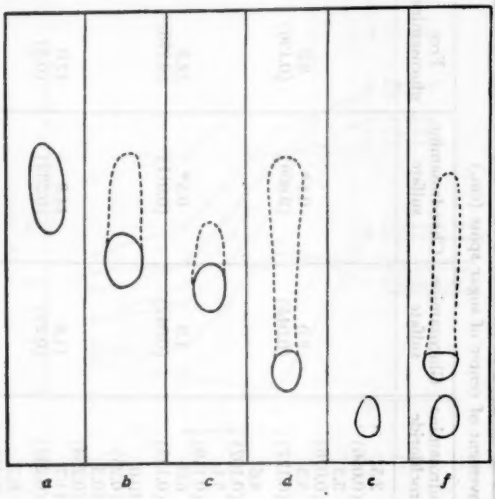

Fig. 3. *n*-Butylacetate-acetic acid-butanol-methanol-water (3 : 2 : 2 : 1 : 1) chromatograms of glucosamine and hexosamine-salts developed with ninhydrin; duration of test 19 hrs., 30°C.

(a) free glucosamine. (b) glucosamine hydrochloride. (c) chondrosamine hydrochloride. (d) glucosamine sulfate. (e) chondrosamine sulfate. (f) sulfates of glucosamine and chondrosamine.

4. Separation of hexuronic acids

Glucuronic, galacturonic and mannuronic acid were run with Solvents 3, 6 and 7 at different temperatures. Table V shows the results together with six figures reproduced from Table III. In Fig. 4 are drawn the chromatograms obtained by Solvent 6 diagrammatically.

In general Rf values rose with temperature. Solvent 7 nearly met the purpose at 20°C but not sufficiently, but Solvents 3 and in particular 6 gave quite remote spots within those limits of temperature. Solvent 6 is pertinent for the purpose, moreover because the spots of hexuronic acids hereby do not fall on those of hexosamines and galactose.

It is further noteworthy that, of those hexuronic acids, glucuronic and mannuronic acid gave a spot of the lactones except when mannuronic acid, within short time after dissolution, was run with Solvent 6. The largeness of the lactone spots increased with the lapse of time after preparing the solutions of the hexuronic acids.

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H. M.

TABLE V

Distances Travelled and Rf Values (in parentheses) of Hexuronic Acids when Run at 20, 25 and 30°C by Solvents 3, 6 and 7

Paper strips 60 cm. × 2.5 cm.; solvents 14 cc. Developed with aniline-phthalic acid reagent of Partridge.

Solvent No.	Composition		Solvent front cm.	Temperature °C	Duration of test hrs.	Movement of center of sugar spots (cm.)				
						Mannuronic acid	Glucuronic acid	Galacturonic acid	Glucurono-lactone	Mannurono-lactone
3	*n*-Butylacetate	3	45	20	20	5.0 (0.111)	4.5 (0.1)	2.0 (0.044)	14.0 (0.311)	12.3 (0.273)
	Acetic acid	2		25	19½	5.8 (0.129)	4.7 (0.104)	2.7 (0.06)	15.5 (0.344)	13.1 (0.291)
	Butanol	2		30	19	7.0 (0.156)	5.0 (0.111)	3.6 (0.08)	15.7 (0.349)	14.0 (0.311)
	Methanol	1								
	Water	1								
6	Amylalcohol	7	40	20	23½	7.0 (0.175)	5.2 (0.13)	0.0 (0.0)	25.5 (0.638)	
	Pyridine	7		25	23	8.0 (0.2)	6.0 (0.15)	0.7 (0.018)	25.6 (0.64)	
	Water	6		30	22½	9.6 (0.24)	7.4 (0.185)	2.0 (0.05)	27.0 (0.675)	
7	Benzylalcohol	3	40	20	23	12.3 (0.308)	11.0 (0.275)	10.1 (0.253)	21.0 (0.525)	21.0 (0.525)
	Acetic acid	3		25	22½	12.7 (0.318)	12.0 (0.3)	11.7 (0.298)	21.8 (0.545)	21.8 (0.545)
	Water	1		30	21½	13.7 (0.343)	12.8 (0.32)	12.3 (0.308)	22.7 (0.568)	22.7 (0.568)

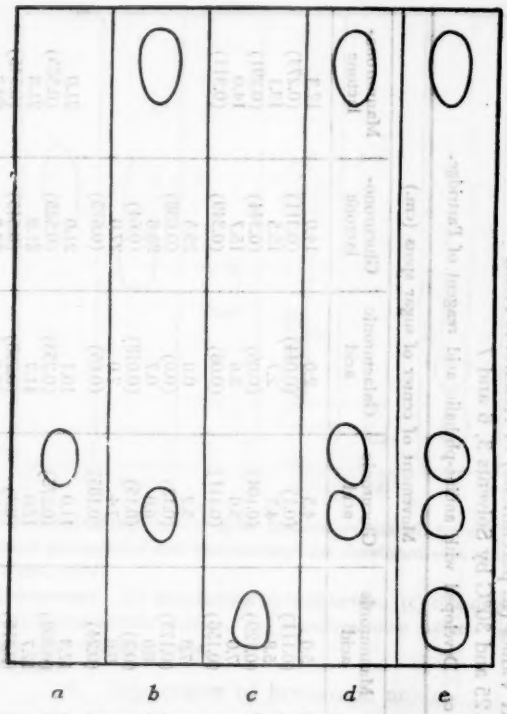

Fig. 4. Amylalcohol-pyridine-water (7:7:6) chromatograms of hexuronic acids developed with aniline-phthalic acid (Partridge); duration of test 22.5 hrs., 30°C.
(a) mannuronic acid. (b) glucuronic acid; the faster moving spot represents glucuronolactone. (c) galacturonic acid. (d) mannuronic and glucuronic acid; the fastest moving spot represents glucuronolactone. (e) mannuronic, glucuronic and galacturonic acid; the fastest moving spot represents glucuronolactone.

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